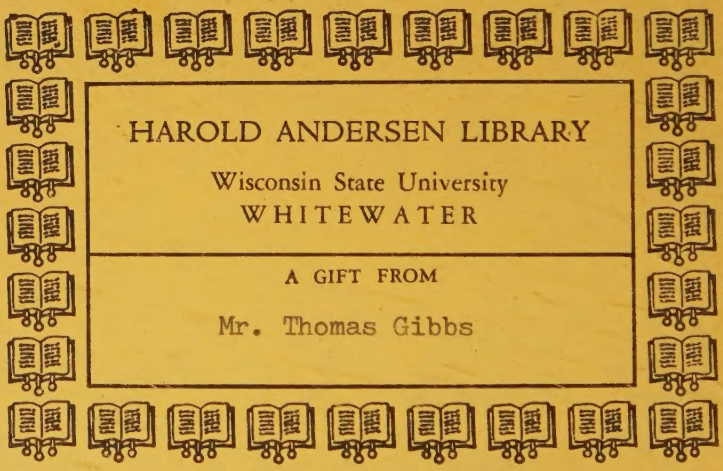
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ELEMENTS

OF

WATER BACTERIOLOGY

WITH SPECIAL REFERENCE TO

SANITARY WATER ANALYSIS

BY

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1915

THIRD EDITION. REWRITTEN

SECOND THOUSAND, REVISED

NEW YORK

JOHN WILEY & SONS, INC.

LONDON: CHAPMAN & HALL, LIMITED

1915

145840

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S. C. PRESCOTT AND C.-E. A. WINSLOW

THE SCIENTIFIC PRESS
ROBERT DRUMMOND AND COMPANY
BROOKLYN, N. Y.

DEDICATED

TO

William Thompson Sedgwick

BY TWO OF HIS PUPILS,

AS A TOKEN OF GRATEFUL AFFECTION

PREFACE TO FIRST EDITION

The general awakening of the community to the importance of the arts of sanitation—accelerated by the rapid growth of cities and the new problems of urban life—demands new and accurate methods for the study of the microbic world. Bacteriology has long since ceased to be a subject of interest and importance to the medical profession merely, but has become intimately connected with the work of the chemist, the biologist, and the engineer. To the sanitary engineer and the public hygienist a knowledge of bacteriology is indispensable.

In the swift development of this science during the last ten years perhaps no branch of bacteriology has made more notable progress than that which relates to the sanitary examination of water. After a brief period of extravagant anticipation, and an equally unreasonable era of neglect and suspicion, the methods of the practical water bacteriologist have gradually made their way, until it is recognized that, on account of their delicacy, their directness, and their certainty, these methods now furnish the final criterion of the sanitary condition of a potable water.

A knowledge of the new science early became so indispensable for the sanitary expert that a special course in the Bacteriology of Water and Sewage has for some years been given to students of biology and sanitary engineering in the Biological Department of the Massachusetts Institute of Technology. For workers in this course the present volume has been especially prepared, and it is fitting, we think, that such a manual should proceed from an institution whose faculty, graduates, and students have had a large share in shaping the science and art of which it treats. We shall be gratified, however, if its field of usefulness extends to those following similar courses in other institutions, or occupied professionally in sanitary work.

The treatment of the subject in the many treatises on General Bacteriology and Medical Bacteriology is neither special enough nor full enough for modern needs. The classic work of Grace and Percy Frankland is now ten years old; and even Horrocks' valuable "Bacteriological Examination of Water" requires to be supplemented by an account of the developments in quantitative analysis which have taken place on this side of the Atlantic.

It is for us a matter of pride that Water Bacteriology owes much of its value, both in exactness of method and in common-sense interpretation, to American sanitarians. The English have contributed researches of the greatest importance on the significance of certain intestinal bacteria; but with this exception the best work on the bacteriology of water has, in our opinion, been done in this country. Smith, Sedgwick, Fuller,

Whipple, Jordan, and their pupils and associates (not to mention others) have been pioneers in the development of this new field in sanitary science. To gather the results of their work together in such form as to give a correct idea of the best American practice is the purpose of this little book; and this we have tried to do with such completeness as shall render the volume of value to the expert and at the same time with such freedom from undue technicality as to make it readable for the layman. It should be distinctly understood that students using it are supposed to have had beforehand a thorough course in general bacteriology, and to be equipped for advanced work in special lines.

Boston, March 10, 1904.

PREFACE TO THIRD EDITION

A SECOND edition of this work was called for in 1908 and it was rewritten in that year, with the inclusion of much new material in the chapters dealing with the isolation of the typhoid bacillus and of intestinal bacteria, and with the addition of a new chapter on the bacteriology of sewage and sewage effluents. In the same year there appeared an excellent volume on Water Bacteriology by Dr. W. G. Savage, which showed the English methods of investigation and interpretation to be closely in accord with those used in America.

In the five years which have elapsed since our second edition was published, there has again been important progress along many lines in sanitary bacteriology; and in particular the publication in 1912 of a new edition of the Report of the Committee on Standard Methods of the Laboratory Section of the American Public Health Association has made necessary a change in many details of current practice.

We have, therefore, prepared at this time a somewhat far-reaching revision of our book. Newer ideas

on the effect of temperature upon the viability of bacteria in water are included in Chapter I. The recent recommendations of the Committee on Standard Methods are discussed in Chapters II and IV; in particular, Chapter IV, dealing with the 37° count, has been expanded. We cannot bring ourselves to agree with the recommendation of the committee that the 37° count should replace the 20° count; but we are entirely in accord with the resolution adopted by the Laboratory Section of the American Public Health Association at its Washington meeting that both determinations should be made in ordinary routine water examinations. Indeed, this is the position we have maintained in both our earlier editions.

Chapter V, dealing with the isolation of specific pathogenes from water, has been extensively rewritten and extended. The use of the Jackson bile medium for the preliminary enrichment of the typhoid bacillus has become general since 1908 and a number of successful isolations have been reported by its use; so that this procedure promises to be of increasing importance in the future.

In regard to the isolation and identification of bacilli of the colon group we feel that the time has come for a change from the usual American practice of the past. The five standard tests for "typical B. coli" established by the Committee on Standard Methods in its 1905 report have come to seem more and more illogical and unscientific to most practical water bacteriologists. The conviction has grown that they go either too far or not far enough. For waters, in the

United States, at least, it seems clear that all of the lactose-fermenting group of bacilli are significant of pollution from human or animal sources when present in considerable numbers. The 1912 report of the Standard Methods Committee apparently takes this view in one place, while retaining the five tests in another section. We have felt it best to place ourselves fairly and fully in line with the view that the whole group of aërobic lactose-fermenting bacilli is significant and this broad definition is the one upon which we have based our general discussion of the colon group in Chapters VI and VII. In Chapter VIII we have discussed the subdivisions of the group as worked out by MacConkey and others and their special significance with respect to recent and remote pollution as suggested by the researches of Houston and Clemesha.

The use of the lactose-bile test as sufficient evidence of the presence of bacilli of the colon group has become very general and was endorsed by the Laboratory Section of the American Public Health Association at its 1912 meeting. The U. S. Treasury Department, acting on the advice of a special commission of experts, in formulating a standard for water supplied by common carriers in interstate commerce has, however, recommended a lactose peptone broth enrichment medium, followed by plate isolation and a confirmatory test in lactose peptone broth. Further study is needed to determine the relative value of these two procedures.

The growing importance of the application of bacteriology to the sanitary study of shellfish has led us to include a new chapter dealing with this subject.

based largely upon the recent report of the Committee of the Laboratory Section of the American Public Health Association.

Throughout the book we have resorted freely to the use of tables of actual data for the illustration of the various points discussed, believing that ample familiarity with practical examples furnishes the only sound basis for judgment in sanitary water examination.

For the benefit of the student the chapters have been sub-divided into sections with prominent headings indicating the general topics under discussion.

Massachusetts Institute of Technology,
BOSTON, Mass.

American Museum of Natural History,
NEW YORK, N. Y.

Feb. 1, 1915.

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ELEMENTS OF WATER BACTERIOLOGY

CHAPTER I

THE BACTERIA IN NATURAL WATERS

Bacteria and Their Nutritive Relations. Bacteria are the most numerous and the most widely distributed of living things. They are present not merely at the surface of the earth or in the bodies of water which partially cover it, as is the case with most other living things, but in the soil itself, and in the air above, and in the waters under the earth.

Probably no organisms are more sensitive to external conditions, and none respond more quickly to slight changes in their environment. Temperature, moisture, and oxygen are of importance in controlling their distribution; but the most significant factor is the amount of food supply. Bacteria and decomposing organic matter are always associated, and for this reason a brief consideration of the general relation of bacteria to their sources of food supply must precede the study of their distribution in any special medium.

The bacteria possess greater constructive ability than any animal organisms. They lack, however,

the power of green plants to build up their own food from compounds like carbon dioxide and nitrates which have no stored potential energy. The food requirements of various bacterial types differ, however, widely among themselves. Fischer (1900) has divided the whole group into three great subdivisions according to the nature of their metabolism. The Prototrophic forms are characterized by minimal nutrient requirements, including organisms like the nitrifying bacteria which require no organic compounds at all, but derive their nourishment from carbon dioxide or carbonates, nitrites and phosphates, or from inorganic ammonium salts. A second group of Metatrophic bacteria includes those forms which require organic matter, nitrogenous and carbonaceous, but are not dependent on the fluids of the living plant or animal. Finally, the Paratrophic bacteria are the true parasites, which exist only within the living tissues of other organisms. These subdivisions, like all groups among the lower plants, are not sharply defined, and the metatrophic bacteria in particular exhibit every gradation, from types which grow in water with a trace of free ammonia to organisms like the colon bacillus which normally occur on the surface of the plant or animal body, feeding upon the fluids or on the extraneous material collected upon its surface.

The vast majority of bacteria belong to the second, or metatrophic group, living as saprophytes on dead organic matter wherever it may occur in nature, and particularly in that diffuse layer of decomposing plant and animal material which we call the humus, or surface

layer of the soil. Wherever there is life, waste matter is constantly being produced, and this finds its way to the earth or to some body of water. The excretions of animals, the dead tissues and broken-down cells of both animals and plants, as well as the wastes of domestic and industrial life, all eventually find their way to the soil. In a majority of cases these substances are not of such chemical composition that they can be utilized at once by green plants as food, but it is first necessary that they go through a decomposition or transformation in which their chemical nature becomes changed; and it is as the agents of this transformation that bacteria assume their greatest importance in the world of life.

We may take the decomposition of a comparatively simple excretory product, urea, as an example of the part which the bacteria play in the preparation of plant food. Through the activity of an enzyme produced by certain bacteria this compound unites with two molecules of water and is converted into ammonium carbonate,

$$CO\begin{matrix}NH_2\\NH_2\end{matrix} + 2H_2O = (NH_4)_2CO_3.$$

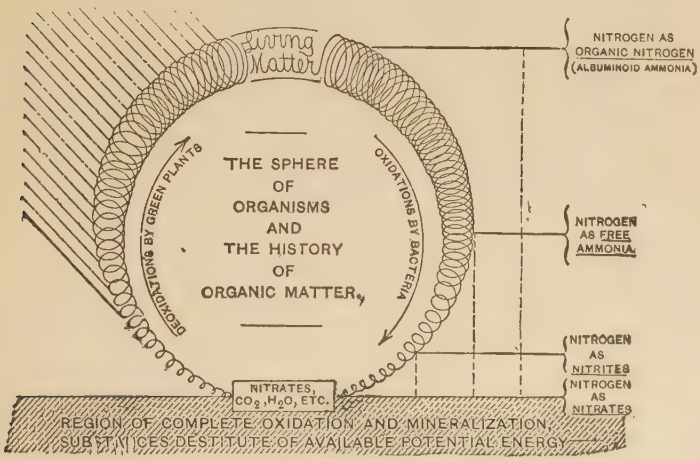
This, however, is only part of the process. While green plants can derive their necessary nitrogen in part, at least, from ammonium compounds it is a well-established fact that this element is often obtained more readily from nitrates, and there are other bacteria which as a further step oxidize the ammoniacal nitro-

gen to a more available form. This process of oxidation is known as *nitrification*, and takes place in a succession of steps, the organic nitrogen being first converted to the form of ammonium salts, and these in turn to *nitrites* and *nitrates*, the oxygen used coming from the air. Several groups of organisms are instrumental in bringing about this conversion. It is generally assumed that one group attacks the ammonium compounds and changes them to nitrites; while another group completes the oxidation to nitrates. In the latter form nitrogen is readily taken up by green plants to be built up into more complex albuminoid substances (organic nitrogen) through the constructive power of chlorophyll.

This never-ending cycle is illustrated in the accompanying figure, devised by Sedgwick (Sedgwick, 1889) to illustrate the transformations of organic nitrogen in nature, the increasing size and closeness of the spiral on the left-hand side indicating the progressive complexity of organic matter as built up by the chlorophyll bodies of green plants in the sunlight, and the other half of the figure the reverse process, carried out largely by the bacteria. In nature there are many short circuits, as, for instance, when dead organic matter is used as food for animals and built up into the living state again without being nitrified and acted upon by green plants; but the complete cycle of organic nitrogen is as indicated on the diagram.

We have dwelt thus at length upon the general relation between bacteria and organic decomposition because in this relation will be found the master key

to the distribution of bacteria in water as well as in other natural habitats. It is true that certain peculiar forms may at times multiply in fairly pure waters; but, in general, large numbers of bacteria are found only in connection with the organic matter upon which they feed. Such organic matter is particularly abundant in the surface layer of the soil. Here, therefore, the bacteria are most numerous; and in other media their


Living Matter
THE SPHERE OF ORGANISMS AND THE HISTORY OF ORGANIC MATTER
REDUCTIONS BY GREEN PLANTS
OXIDATIONS BY BACTERIA
NITROGEN AS ORGANIC NITROGEN (ALBUMINOID AMMONIA)
NITROGEN AS FREE AMMONIA
NITROGEN AS NITRITES
NITROGEN AS NITRATES
NITRATES, CO_2, H_2O, ETC.
REGION OF COMPLETE OXIDATION AND MINERALIZATION
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numbers vary according to the extent of contact with the living earth.

Classification of Waters. Natural waters, then, group themselves from a bacteriological standpoint in four well-marked classes, according to their relation to the rich layers of bacterial growth upon the surface of the globe. There are first the *atmospheric waters* which have never been subject to contact with the earth; second, the *surface-waters* immediately exposed to such

contamination in streams and pools; third, *stored waters* in lakes and large ponds where storage has reduced bacterial numbers and produced a state of comparative purity; and fourth, the *ground-waters* from which previous contamination has been even more completely removed by filtration through the deeper layers of the soil.

Bacterial Content of Various Waters. Even rain and snow, the sources of our potable waters, are by no means free from germs, but contain them in numbers varying according to the amount of dust present in the air at the time of the precipitation. After a long-continued storm the atmosphere is washed nearly free of bacteria, so that a considerable series of sterile plates may often be obtained by plating 1-c.c. samples. These results are in harmony with the observations of Tissandier (reported by Duclaux, 1897), who found that the dust in the air amounted to 23 mg. per cubic meter in Paris and 4 mg. in the open country. After a rainfall these figures were reduced to 6 mg. and 0.25 mg., respectively.

With regard to what may be considered normal values for rain it is difficult to give satisfactory figures. Those obtained by Miquel (Miquel, 1886) during the period 1883–1886 showed on the average 4.3 bacteria per c.c. in the country (Montsouris) and 19 per c.c. in Paris. Snow shows rather higher numbers than rain. Janowski (Janowski, 1888) found in freshly fallen snow from 34 to 463 bacteria per c.c. of snow-water.

As soon as the rain-drop touches the surface of the earth its real bacterial contamination begins. Rivulets from ploughed land or roadways may often contain several hundred thousand bacteria to the cubic centi-

meter; and furthermore the amounts of organic and mineral matters which serve as food materials, and thus become a factor in later multiplication of organisms, are greatly increased.

In the larger streams several conditions combine to make these enormous bacterial numbers somewhat lower. Ground-water containing little microbic life enters as a diluting factor from below. The larger particles of organic matter are removed from the flowing water by sedimentation; many earth bacteria, for which water is an unfavorable medium, gradually perish; and in general a new condition of equilibrium tends to be established. It is difficult, however, to find a river in inhabited regions which does not contain several hundreds or thousands of bacteria to the cubic centimeter. Furthermore, heavy rains which introduce wash from the surrounding watershed may at any time upset whatever equilibrium exists, and surface-waters are apt to show sudden fluctuations in their bacterial content.

Seasonal Variation of Bacteria in Surface Waters. Sharp variations in bacterial content are particularly apt to occur in the spring and fall as a result of the rain and melting snow at those seasons. The high numbers shown for various rivers in the table on page 8 illustrate this point.

The rainfall is the main factor which causes these seasonal variations; but its specific effect differs with different streams. The immediate result of a smart shower is always to increase contamination by introducing fresh wash from the surface of the ground. More prolonged

SEASONAL VARIATIONS IN BACTERIAL CONTENT OF RIVER WATERS. BACTERIA PER C.C. MONTHLY AVERAGE

River.	Year.	Jan.	Feb.	Mar.	April.	May.	June.
Thames [1]	1905–6	2,075	1,679	1,161	277	1,064	382
Lea [1]	1905–6	5,192	3,083	1,308	471	1,350	598
New [1]	1905–6	1,455	1,304	291	149	352	198
Mississippi [2]	1900–01	972	2,871	1,795	3,597	2,152	2,007
Potomac [3]	1906–7	4,400	1,000	11,500	3,700	750	2,300
Merrimac [4]	1905	14,200	14,800	10,300	3,600	1,900	9,600
Susquehanna [5]	1906	9,510	21,228	31,326	39,905	6,187	2,903

River.	Year.	July.	ug.	Sept.	Oct.	Nov.	Dec.
Thames [1]	1905–6	952	....	....	....	1,633	740
Lea [1]	1905–6	1,190	....	....	....	3,946	2,050
New [1]	1905–6	450	....	....	....	718	621
Mississippi [2]	1900–01	1,832	805	....	....	....	2,021
Potomac [3]	1906–7	2,700	3,000	6,200	2,300	1,800	6,900
Merrimac [4]	1905	3,900	19,500	13,500	39,800	8,700	.....
Susquehanna [5]	1906	685	1,637	836	7,575	26,224	37,525

[1] Houston, 1906*a*, 1906*b*.
[2] New Orleans, 1903.
[3] Figures obtained through courtesy of F. F. Longley.
[4] Massachusetts, 1906.
[5] Harrisburg, 1907.

moderate rain, however, exerts an opposite effect, and after the main impurities which can be washed away have been removed, may dilute the stream with water purer than itself. What the net effect of rain may be depends, therefore, on the character of the stream. A river of fairly good quality shows its highest numbers in rainy periods. With a highly polluted stream, on the other hand, the constant influx of sewage overbalances occasional contributions of surface contamination. Thus Gage (1906) shows in the following table that the bacterial content of the Merrimac is highest when the stream is lowest, that is, when its sewage content is least subject to dilution.

VARIATIONS IN BACTERIAL CONTENT, MERRIMAC RIVER

GAGE (1906)

Flow of Stream. Cubic Feet per Second per Square Mile of Watershed	Bacteria per c.c.		B. coli per c.c.	
	Canal.	Intake.	Canal.	Intake.
Less than 1	7,500	10,800	66	88
1–2	6,800	6,200	50	51
2–4	3,600	5,600	29	39
Over 4	3,400	3,100	16	29

The contrast between the two classes of rivers is well brought out in a study of the Lahn and the Wieseck, by Kisskalt (1906); and the table below, compiled from his data, gives an excellent idea of the total numbers of bacteria and their seasonal fluctuations in a stream of fair quality (the Lahn) and a highly polluted one (the Wieseck). In the former case the bacterial numbers are highest when rain brings surface pollution; in the latter, when the sewage constantly present is least diluted.

MONTHLY VARIATIONS OF BACTERIA IN A NORMAL AND POLLUTED STREAM

KISSKALT, 1906

Date.	Bacteria per c.c.		Date.	Bacteria per c.c.	
1904.	Lahn.	Wieseck.	1904–5.	Lahn.	Wieseck.
July	318	104,000	December [1]	1,220	21,200
July	132	156,800	January [1]	3,668	29,920
August	840	98,400	February [1]	5,380	11,900
October [1]	1,235	28,400	March [1]	1,210	8,250
October [1]	420	58,000	April [1]	4,925	5,910
November	2,340	39,200	May	570	14,800
November [1]	1,740	52,000	June	686	50,180
December [1]	780	28,600			

[1] Rain or high water due to previous thaw.

Effect of Storage upon Bacteria in Water. In standing waters all the tendencies which make for the reduction of bacteria are intensified, and when a river passes into a natural or artificial reservoir a notable reduction in numbers occurs. The table below shows the striking effect produced upon the water of the Potomac River by its successive passage through the three reservoirs of the Washington water supply in the first nine months of 1907. We owe these figures to the courtesy of Mr. F. F. Longley, the engineer then in charge of the Washington filter plant.

REDUCTION OF BACTERIA IN WASHINGTON RESERVOIRS. BACTERIA PER C.C., MONTHLY AVERAGE, 1907

	Potomac River.	Dalecarlia Reservoir.	Georgetown Reservoir.	Washington City Reservoir.
January	4,400	2,400	2,200	950
February	1,000	950	1,000	750
March.....	11,500	8,300	7,200	3,600
April.....	3,700	2,100	1,400	475
May.....	750	350	325	130
June.....	2,300	950	600	100
July.....	2,700	600	350	160
August.....	3,000	275	425	80
September ...	6,200	.....	1,900	230

The still more striking results obtained at London are indicated in the table on page 11.

When the water which enters a pond or a reservoir has already undergone considerable storage and reached a comparatively stable condition, the diminution due to additional storage may be almost negligible. Thus Philbrick (1905) found that the influent water of the Chestnut Hill Reservoir of the Metropolitan Water

Works of Boston contained on the average during the eleven years, 1893–1903, 220 bacteria per c.c., and the effluent 179. In many individual months, and in some whole years, the effluent contained more than the influent.

AVERAGE REDUCTION OF BACTERIA BY STORAGE AT LONDON

(HOUSTON, 1909)

Water	Storage, Days.	Bacteria per c.c.		
		Gelatin 20°.	Agar 37°.	Bile-salt Agar 37°.
Raw Thames River	.....	4465	280	41
Do. stored at Staines	95	175	34	2
Do. stored at Chelsea	15	208	44	5
Do. stored at Lambeth	14	362	52	8
Raw Lee River	.....	8135	382	34
Do. stored	58	67	11	1

The seasonal variations in the bacterial content of a large pond or lake follow a somewhat different course from those observed in a stream. Philbrick, in the paper just cited, gives the figures tabulated below for the Chestnut Hill Reservoir of the Metropolitan Water Works (Boston). The averages are based on weekly analyses covering the eleven years, 1893–1903.

MONTHLY VARIATIONS IN BACTERIAL CONTENT OF CHESTNUT HILL RESERVOIR, 1893–1903

Month.	J.	F.	M.	A.	M.	J.	J.	A.	S.	O.	N.	D.
Bacteria per c.c.	82	73	71	123	69	73	82	95	134	89	103	96

The marked increase in April and September is the notable feature of these analyses; and this is due to the effect of the spring and fall overturns which, in the months in question, stir up the decomposing organic matter at the bottom and distribute it through the reservoir. The slight, but steady, increase during the warm months from May to August is also probably significant.

On the whole it may be said that the bacterial content of a lake or pond should not be more than one or two hundred per c.c. and may often be under a hundred. The student will find numerous analyses of natural waters in Frankland's classic work (Frankland, 1894). He notes, for example, that the Lake of Lucerne contained 8 to 51 bacteria per c.c., Loch Katrine 74, and the Loch of Lintralthen an average of 170. The water of Lake Champlain examined by one of us (S. C. P.) in 1896 contained on an average 82 bacteria per c.c. at a point more than two miles out from the city of Burlington. Certain surface water-supplies near Boston studied by Nibecker and one of us (Winslow and Nibecker, 1903), gave the following results:

City.	Number of Samples.	Average Number of Bacteria per c.c.
Wakefield	7	59
Lynn	6	16
Plymouth	6	35
Cambridge	5	94
Salem	5	232
Medford	5	524
Taunton	4	13
Peabody	3	141

In sea-water, too, bacterial numbers are small, as noted by Russell at Naples (Russell, 1891) and Wood's Hole (Russell, 1892), and in salt as in fresh water the amount of bacterial life decreases in general as one passes downward from the surface and outward from the shore. Otto and Neumann (1904) obtained the results summarized below at various points on the high seas between Portugal and Brazil. Near the European coast numbers were much higher.

BACTERIA IN THE ATLANTIC OCEAN. (OTTO AND NEUMANN, 1904.) BACTERIA PER C.C.

Nearest Land.	Depth in Meters.			
	5	50	100	200
Canary Islands	120	76	20	1
Cape Verde Islands	58	16	64	6
St. Paul Island	20	480	54	4
Pernambuco	48	168	83	14

Drew (1912) finds high numbers of bacteria in surface sea-water off the Bahamas, ranging from 13,000 to 16,000, falling off below 200 fathoms (in the cold bottom waters at 10° C. or below) to 0 to 17.

Factors Influencing the Diminution of Bacteria in Surface-waters. The decrease in numbers which takes place when a surface-water is stored in a pond or reservoir indicates that the forces which tend to produce bacterial self-purification are important ones. It is necessary to consider in somewhat more detail just what these forces are, in order to gauge their potency in any particular instance.

Chief of them appear to be sedimentation, the activity of other micro-organisms, light, temperature, and food-supply, and perhaps more obscure conditions such as osmotic pressure.

The subsidence of bacteria, either by virtue of their own specific gravity, or as the result of their attachment to particles of suspended matter, is unquestionably partly, if not largely, responsible for changes in the number of bacteria in the upper layers of water at rest or in very sluggish streams. The results of numerous investigations by different workers seem to indicate that sedimentation of the bacteria themselves takes place slowly, and that the difference in numbers between the top layer and the bottom layer of water in tall jars in laboratory experiments of a few days' duration is very slight or quite within the limits of experimental error (Tiemann and Gärtner, 1889). Different species may, of course, be differently affected (Scheurlen, 1891). It must be remembered, however, that in natural streams bacteria are to a great extent attached to larger solid particles upon which the action of gravity is more important. Spitta (1903) found that from one-fifth to one-half of the bacteria in canal water may be attached to gross particles, as evidenced by their sedimentation in a few hours. Jordan (Jordan, 1900) is firmly of the opinion that in the lower part of the Illinois River, where there is a fall of but 30 feet in 225 miles, the influences summed up by the term sedimentation are sufficiently powerful to obviate the necessity for summoning another cause "to explain the diminution in numbers of bacteria," and he further

adds: "It is noteworthy that all the instances recorded in the literature where a marked bacterial purification has been observed are precisely those where the conditions have been most favorable for sedimentation."

Little is known as to the share of other organisms in hastening the decrease of bacteria in stored water. Doubtless predatory Protozoa play some part in the process. Huntemüller (1905) after infecting water containing flagellate Protozoa with typhoid bacilli, found the Protozoa crowded with bacteria; and he observed under the microscope the actual ingestion of the living and motile bacilli. Korschun (1907) and others have obtained similar results and consider the activity of Protozoa to be an important factor in self-purification. Fehrs (1906) found that typhoid bacilli would live for 7 days in unsterilized Göttingen tap water, for 46 days in the same water sterilized, and for 13 days in water inoculated with a culture of flagellate Protozoa after sterilization. Water bacteria were of course added with the Protozoa. Stokvis and Swellengrebel (1911) have shown that ciliated infusoria may also consume considerable quantities of bacteria under favorable conditions as to oxygen and temperature, and Hörhammer (1911) reports that certain Crustacea such as Cyclops may devour considerable quantities of typhoid bacilli when present in masses from cultures, stained with methylene blue, and suspended in water.

Certain bacteriologists have held that the toxic waste products of the bacteria themselves may render water unfit for their own development. Horrocks (Horrocks, 1901), Garré (Garré, 1887), Zagari (Zagari, 1887) and

Freudenreich (Freudenreich, 1888) have shown that an "antagonism" exists when bacteria are grown in artificial culture media, such that the substratum which has supported the growth of one form may be rendered antiseptic to another. Frost (1904) has exhaustively studied the phenomenon of antagonism by exposing typhoid bacilli in collodion sacs to the action of certain soil and water bacteria growing in broth. Artificial culture media, however, offer conditions for bacterial development which are scarcely paralleled in natural waters. It is difficult to believe that under ordinary conditions poisons are produced of such power as to render a stream or lake specifically toxic for any particular type of bacteria. It does appear indeed from the experiments of Jordan, Russell and Zeit (1904), and Russell and Fuller (1906), which will shortly be referred to more fully, that the life of typhoid germs is shorter in water containing large numbers of other bacteria than in that of greater purity. Horrocks (1899), too, found freshly isolated typhoid bacilli alive in sterile sewage after 60 days; while they disappeared in 5 days when B. coli was also present. These phenomena may be due, however, to a struggle for oxygen, or for food, rather than to the assumed presence of highly toxic bacterial products, of which there is no independent evidence.

Many investigations conducted since the pioneer researches of Downes and Blunt (Downes and Blunt, 1877) have confirmed the results reported by them, which showed that direct sunlight is fatal to most bacteria in the vegetative state and even to spores if

the exposure be sufficiently long, while diffused light is harmful in a less degree. Opinions vary as to the degree to which light is active in destroying the bacteria in natural waters. Buchner (Buchner, 1893) found by experiment that the bactericidal power of light extends to a depth of about three meters before it becomes imperceptible. On the other hand, Procaccini (Procaccini, 1893) found that when sunlight was passed vertically through 60 cm. of drain-water the lower layers contained nearly as many bacteria after 3 hours' treatment as before the exposure. The middle and upper portions showed a great falling off in numbers, however.

But few studies have been made of the effect of light on bacteria in flowing water. Jordan (Jordan, 1900) has investigated several Illinois streams and arrived at the conclusion that in moderately turbid water, at least, the sun's rays are virtually without action. On the other hand, Rapp has observed a considerable reduction of the bacteria in the Isar at Pullach after the period of diurnal insolation, as shown by the table on the following page. Clemesha (1912[a]) attributes very great importance to the action of light in the self-purification which takes place in Indian lakes and rivers; his opinion is apparently not based on comparative experiments including and excluding this factor, but chiefly on the greater numbers of intestinal bacteria at the bottom as compared with the superficial layers of water.

It is unnecessary to dwell in detail upon the effect which the lack of nutritive elements must exert upon

intestinal bacteria and soil bacteria in waters of ordinary purity. Comparative studies of culture media, to be quoted in the succeeding chapter, will show how delicately the bacteria respond to comparatively slight changes in their food-supply. Wheeler (1906) found that typhoid bacilli would persist in almost undiminished numbers in sterilized water from a polluted well containing considerable organic matter and kept in the dark at 20 degrees, while in purer water or in the light they died out in from 2 to 6 weeks.

EXAMINATIONS OF THE ISAR AT PULLACH

(RAPP, 1903)

(*A*) *Carried out September 26, 1898, no rain having fallen for three weeks*

Temperature.		Time o the Experiment.	Bacteria per c.c.
of the Water.	of the Air.		
13.0° C.	8.8° C.	7.30 P.M.	146
12.1° C.	7.0° C.	9.30 P.M.	270
10.5° C.	6.2° C.	5.00 A.M.	370
10.2° C.	8.2° C.	8.00 A.M.	320

(*B*) *Carried out November 28, 1898, no rain having fallen for some time*

5.5° C.	3.0° C.	6.00 P.M.	266
5.5° C.	2.5° C.	8.00 P.M.	402
5.5° C.	2.0° C.	10.00 P.M.	482
5.0° C.	2.0° C.	3.00 A.M.	532
4.5° C.	2.5° C.	7.30 A.M.	400

Whipple and Mayer (1906) have called attention to another important factor in the general problem. They find that the presence of oxygen is essential to the per-

sistence of typhoid and colon bacilli in water, although in nutrient media both forms may thrive under anærobic conditions.

EFFECT OF OXYGEN ON VIABILITY OF TYPHOID BACILLI IN STERILE TAP WATER

WHIPPLE AND MAYER, 1906

Period in Days	Tubes Kept in Air.		Tubes Kept in Hydrogen.	
	Bacteria per c.c.	Per Cent.	Bacteria per c.c.	Per Cent.
0	600,000	100.0	600,000	100.0
2	455,000	76.0	2,400	0.4
4	190,000	32.0	25	0.004
8	120,000	20.0	0	0.0
12	67,000	11.0	0	0.0
18	25,000	4.2	0	0.0
26	9,250	1.5	0	0.0
33	2,150	0.6	0	0.0
40	132	0.02	0	0.0
47	6	0.001	0	0.0
54	0	0.000	0	0.0

Various inorganic constituents of the medium undoubtedly exercise an important influence upon the life of bacteria in water; and the mutual interaction of the different substances present is a highly complex one. Thus Winslow and Lochridge (1906) report that five parts of dissociated hydrogen per million parts of tap water (0.005 normal HCl) is fatal to typhoid bacilli, while ten times as much acid is required for sterilization when 1 per cent of peptone is present to check the dissociation of the hydrogen. In Hazen and Whipple's study of the Allegheny, Monongahela and Ohio rivers

at Pittsburgh the antiseptic effect of acid wastes was strikingly shown. (*Engineering News*, 1912.)

Although it is hard to estimate the exact importance of each factor, the general phenomena of the self-purification of streams are easy to comprehend. A small brook, immediately after the entrance of polluting material from the surface of the ground, contains many bacteria from a diversity of sources. Gradually those organisms adapted to life in the earth or in the bodies of plants and animals die out, and the forms for which water furnishes ideal conditions survive and multiply. It is no single agent which brings this about, but that complex of little-understood conditions which we call the environment. If any one thing is of prime importance it is probably the food-supply, for only certain bacteria are able to multiply in the presence of the small amount of organic matter present in ordinary potable waters. As Jordan (Jordan, 1900) has said: "In the causes connected with the insufficiency or unsuitability of the food-supply is to be found, I believe, the main reason for the bacterial self-purification of streams."

Effect of Temperature upon Bacteria in Water. The effect of temperature upon the survival of bacteria in water varies according to this primary condition of food-supply which has just been discussed. When bacteria are in a medium in which they are able to grow and multiply, warmth, within reasonable limits of course, favors their development. At times this may be true even of certain intestinal bacteria in water. Thus at Harrisburg, Pa., a series of B. coli examinations

made in the midsummer of 1906 showed positive results in 7 per cent of the samples of water entering the storage reservoir and in 27 per cent of the samples leaving it. The storage period in this case was about two days and the temperature of the water in the reservoir was nearly at blood heat (Harrisburg, 1907). Clemesha (1912[a]) has recently made an exhaustive study of this multiplication of coli-like microbes in warm waters and has shown that it is confined to certain particular types within the colon group. For most intestinal bacteria the conditions necessary for growth and multiplication are not realized in water and an entirely different temperature effect is manifest. When a bacterium cannot multiply, the only vital activity which can take place is a katabolic wasting away, which soon proves destructive, and the higher the temperature the more rapidly the fatal result is reached. A frog in winter lives at the bottom of a pond breathing only through its skin and eating not at all, but as soon as the temperature rises it must eat and breathe through its lungs or perish. It is quite true that even in ice 40 per cent of typhoid bacilli perish in 3 hours and 98 per cent in 2 weeks (Sedgwick and Winslow, 1902). Recent work has shown, however, that they die in spite of the cold, not on account of it, and that the decrease is more rapid at higher temperatures, unless of course food-supply and other conditions admit of multiplication. Houston (1911) has furnished a very clear demonstration of this temperature relation by storing typhoid bacilli in water with the results tabulated on page 22.

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EFFECT OF TEMPERATURE ON SURVIVAL OF TYPHOID BACTERIA IN WATER

(HOUSTON, 1911)

Temperature C.	Percentage of Typhoid Bacilli Surviving after One Week.	Period of Final Disappearance of Bacilli.
0	46	9 weeks
5	14	7 weeks
10	0.07	5 weeks
18	0.04	4 weeks

Ruediger (1911) has shown that colon bacilli are far more abundant in the Red Lake River during the winter when the river is covered with ice than in summer, although the volume of the river and the amount of sewage pollution are about the same. Typhoid bacilli in celloidin dialyzers floated down the river showed only 2.5 and 3.5 per cent surviving in 2 days and 0.51, 0.89, 2.2 and 3.2 per cent surviving in 3 days when the river was not frozen, while dialyzers suspended through the ice in colder weather showed 6.1, 10.5, 17.7, 46.8 and 62.9 per cent surviving in five different experiments after 2 days, 31 per cent in 3 days, 19 per cent in 7 days, and 2.5 per cent in 14 days. Ruediger attributes this greater persistence at low temperatures to the absence of poisonous waste products of other organisms and to protection from the light; but there can be little doubt that it is mainly a result of the general preservative effect of cold. From an epidemiological standpoint the conclusion that disease germs perish quickly in warm waters is amply confirmed. Almost without exception outbreaks of typhoid fever due to polluted water occur in cold weather and this is, in

part at least, due to the greater persistence of typhoid bacilli at low temperatures.

Relation between Time of Storage and Self-purification. It is obvious that the efficiency of all the agencies which tend to decrease the number of bacteria in surface waters will increase with the prolongation of the period for which they act. Time is the great measure of self-purification; and in a comprehensive study of the self-purification of the Potomac River, the investigators of the U. S. Public Health Service have found that the time relation is close enough to be stated mathematically. Prof. Phelps expresses this in the formula $\log \frac{n_1}{n_2} = kt$, where t is the time and n_1 and n_2 the respective numbers of bacteria; k, of course, will vary with the temperature and the other conditions discussed above.

The absolute time required to make water safe will vary widely with this value k. Jordan, Russell and Zeit (1904), in an important series of experiments, added typhoid bacilli to the unsterilized waters of Lake Michigan, the Chicago River and Drainage Canal and the Illinois River, in collodion sacs suspended in the respective bodies of water. From the relatively pure Lake Michigan water the specific organisms could be isolated for at least a week, but in the polluted waters of the rivers and the Drainage Canal they were not found after 3 days except in a single instance. Russell and Fuller, (1906) confirmed these general results, finding that typhoid bacilli would live for 10 days in the unsterilized water of Lake Mendota, while they could be isolated only after 5 days when immersed in sewage. Other observers record much greater

viability for the typhoid bacillus. Savage (1905) added a heavy dose of the organism to unsterilized tidal mud and found it living after 5 weeks. Hoffmann (1905), after inoculating a large aquarium with a rich typhoid culture, was able to isolate the germ from the water after four weeks and from the mud at the bottom after two months. Konrádi (1904) reports the persistence of typhoid bacilli in unsterilized tap water for over a year.

These last experiments deal only with the maximum survival period for a few out of great numbers of germs introduced into the water or mud, and entirely ignore the quantitative aspects of the case. When one considers the proportion of the original bacteria surviving, the period necessary to bring about a reasonably safe condition is found to be much shorter. Houston (1908) has shown that when water is artificially infected with typhoid bacilli and stored, 99.9 per cent of the disease germs perish in one week, although some may persist for from 1 to 9 weeks.

In later experiments (Houston, 1911) he finds that "uncultivated" typhoid bacilli added to the water directly from the urinary sediment of a disease carrier perish much more rapidly than the laboratory strains, usually disappearing entirely after one week and always after three. On a number of occasions Dr. Houston gave dramatic expression to his confidence in these negative laboratory findings by drinking half pint portions of water which a few weeks previously had contained millions of typhoid bacilli. We have plenty of practical epidemiological evidence, such as that

offered in the Chicago Drainage Canal case and in the lawsuit over the condition of the water supply of Jersey City, to confirm the general conclusion that any water which has been stored for 4 weeks is practically safe.

Bacteria in Ground-waters. In general we have seen that surface-waters tend continually to decrease in bacterial content after their first period of contact with the humus layer of the soil. In that other portion of the meteoric water which penetrates below the surface of the earth to join the reservoir of ground-water, later to reappear as the flow of springs and wells, this diminution is still more marked, since the filtering action of the earth removes not only most of the bacteria, but much of their food material as well. The numbers of bacteria in the soil itself decrease rapidly as one passes downward. Kabrhel (1906) found several million per c.c. in surface samples of woodland soil, a few thousands or tens of thousands half a meter below, and usually only hundreds in centimeter samples collected at depths greater than a meter. Many observers formerly believed that all ground-waters were nearly free from bacteria, because often no colonies appeared on plates counted after the ordinary short periods of time. If, however, a longer period of incubation be adopted considerable numbers may be obtained.

For convenience we may divide ground-waters into three groups, namely: shallow open wells, springs and "tubular" (driven) or deep wells. This division is important because ordinary shallow wells form a group by themselves in respect to the possibility of aerial and

surface contamination, their water often being fairly rich in bacterial life. Egger (Wolffhügel, 1886) examined 60 wells in Mainz and found that 17 of them contained over 200 bacteria to the cubic centimeter. Maschek (Maschek, 1887) found 36 wells out of 48 examined in Leitmeritz which had a bacterial content of over 500 per c,c. Fischer (Horrocks, 1901) reported 120 wells in Kiel which gave over 500 bacteria per c.c. and only 51 with less than that number.

In the examination of 147 shallow farmyard wells by one of us (S. C. P.) it was found that 124 of the wells which contained no B. coli, and were therefore probably free from fecal pollution, averaged 190 bacilli per c.c. while 23 which gave positive tests for B. coli averaged 570 per c.c. The distribution of the two series of samples according to the number of bacteria present is indicated in the table below.

BACTERIA IN SHALLOW FARMYARD WELLS

PERCENTAGE OF SAMPLES IN EACH GROUP

Bacteria per c c	0	1–10	11–20	21–50	51–100	101–500	501–1000	1001–2000	2001–3000
Series I. B. coli absent.	3	16	14	16	11	31	5	4	.....
Series II. B. coli present.	..	..	5	...	10	57	10	14	5

Very similar results are reported for shallow wells used as farm water-supplies in Minnesota by Kellerman and Whittaker (1909), although the general quality of the wells examined was considerably below that of the series tabulated above.

In the ordinary standard 48-hour period very few bacteria develop from normal spring-waters. Thus in an examination of spring-waters made by the Massachusetts State Board of Health in 1900 (Massachusetts State Board of Health, 1901), of 37 springs which were practically unpolluted and had less than 0.10 part per 100,000 excess of chlorine over the normal, 54 samples were examined and gave an average of 41 bacteria per c.c. Only 6 samples showed figures over 50.

It now remains to consider the other great division of ground-waters, namely, deep, " driven," or " tubular " wells, which, if carefully constructed, should ordinarily be free from all surface-water contamination, and should show low bacterial counts. The results tabulated below obtained by Houston in the examination of a series of deep wells of high quality at Tunbridge Wells are fairly typical.

BACTERIAL CONTENT OF DEEP WELL WATERS

(Houston, 1903)

Bacteria per c.c.

36	6	9	4	1
16	17	4	3	12
2	4	10	5	2

Fifteen driven wells in the neighborhood of Boston, examined in 1903, showed at the end of 48 hours an average of only 18 colonies per c.c.; and the results of certain examinations of other wells and springs, recently made by the authors, are given in the table on page 28.

BACTERIA IN DEEP WELL AND SPRING WATERS

Town	Bacteria per c.c.	Town.	Bacteria per c.c.
Worcester, Mass.....	10	Saranac Lake. N. Y. .	11
Waltham, Mass.....	3	Ellenville, N. Y.....	0
Newport, R. I.....	7	Hyde Park, Mass.....	12

It is plain that water absolutely free from bacteria is not ordinarily obtained from any source. In deep wells, however, their number is small; and the peculiar character of the organisms present is manifested in many cases by the slow development at room temperature (frequently no growth until the third day), the entire absence of liquefying colonies, and the abundance of chromogenic species.

CHAPTER II

THE QUANTITATIVE BACTERIOLOGICAL EXAMINATION OF WATER

Relation of the Medium to the Number of Bacteria Obtained. The customary methods for determining the number of bacteria in water do not reveal the total bacterial content, but only a very small fraction of it, as becomes apparent when we consider the large number of organisms, nitrifying bacteria, strict anærobes, etc., which refuse to grow, or grow only very slowly in ordinary culture media, and which, therefore, escape detection. On the one hand, certain obligate parasites cannot thrive in the absence of the rich fluids of the animal body; on the other hand, the prototrophic bacteria, adapted to the task of wrenching energy from nitrites and ammonium compounds are unable to develop in the presence of so much organic matter. Winslow (1905) in the examination of sewage and sewage effluents, found 20–70 times as many bacteria by microscopic enumeration as by the gelatin plate count. Certain special media enable us to obtain much larger counts than those yielded by the ordinary gelatin method. The Nährstoff Heyden agar, for example, has been strongly advocated by Hesse (Hesse and Niedner, 1898) and other German bacteriologists upon this

ground. In this country Gage and Phelps (Gage and Phelps, 1902) showed that the numbers obtained by the ordinary procedure were only from 5 to 50 per cent of those obtained by the use of Heyden's Nährstoff agar. For practical sanitary purposes, however, our methods are fairly satisfactory. Within limits, it is of no great importance that one method allows the growth of more bacteria than another. When we are using the quantitative analysis as a measure of sewage pollution the essential thing is that the section of the total bacterial flora which we obtain should be thoroughly representative of that portion of it in which we are most interested—the group of the quickly growing, rich-food-loving sewage forms. In this respect meat-gelatin-peptone appears to be unrivalled; and it is in this respect that such media as Nährstoff agar fail. Müller (1900) showed that the larger counts obtained by plating on the Nährstoff medium are due to the fact that it specially favors the more prototrophic forms, among the water bacteria themselves. Intestinal organisms and even the ordinary putrefactive germs, when plated in pure culture, show no higher counts on Nährstoff agar than on gelatin. Gage and Adams (1904) found by plating pure cultures of the common laboratory bacteria, saprophytes and parasites, that Nährstoff counts were actually lower than those obtained by the use of gelatin. When sewage and highly polluted waters are examined counts are slightly higher on Nährstoff media, while with purer waters the Nährstoff numbers are far in excess of those obtained with gelatin. Winslow (1905) found the ratio of Nährstoff agar

to gelatin count to be 1.7 to 1.0 for sewage, and 4.8 to 1.0 for sand filter effluent. With waters of still better quality the ratio goes up higher, reaching a maximum when the bacteria which increase and multiply in water are most abundant. Müller (1900) found, for example, that water which normally showed six times as many bacteria on Nährstoff agar as on gelatin might give a Nährstoff-gelatin ratio of 20–30 after it had been standing for some time in the supply pipes. The table below, taken from the valuable paper by Gage and Phelps (1902), shows strikingly the different Nährstoff-agar ratios for waters of

TABLE SHOWING PERCENTAGES OF BACTERIA DEVELOPING ON REGULAR AGAR AND NÄHRSTOFF AGAR FOR DIFFERENT CLASSES OF WATERS

(Gage and Phelps, 1902)

Regular Agar

Class of Water.	Days' Count.						
	2	3	4	5	6	7	8
Ground water.....	0	5	6	6	6	6	6
Filtered water.....	6	7	7	7	7	7	7
Merrimac River...	6	7	7	8	8	9	9
Filtered sewage....	14	17	18	19	19	19	19
Sewage.....	34	44	46	46	46	46	46

Närhstoff Agar

Ground water.....	6	43	78	88	93	100	100
Filtered water.....	37	69	80	92	98	100	100
Merrimac River...	29	78	93	97	97	99	100
Filtered sewage....	26	65	93	95	97	99	100
Sewage.....	39	75	95	100	100	100	100

various grades of purity. It is obvious from all these facts that the effect of using the Nährstoff medium is to increase disproportionately the bacterial counts obtained from purer waters and thus to diminish the difference in bacterial content between normal and contaminated sources. The ordinary agar and gelatin media, on the other hand, are adapted to the growth of intestinal and putrefactive forms and, therefore, serve best the prime object of bacteriological water examination.

The first requisite in a procedure for water analysis is, then, that it should be adapted to the end in view, the differentiation of pure and contaminated waters. The second and equally important requirement is that the procedure should be a standard one, so that results obtained at different times and by different observers may be comparable. In this respect the work of G. W. Fuller, G. C. Whipple, and other members of the Committee on Standard Methods of the American Public Health Association has placed the art of quantitative water analysis in this country in a very satisfactory state by contrast with the varying practices which prevail in England and Germany. The first report on this question was made in 1897 (Committee of Bacteriologists, 1898). A permanent Committee on Standard Methods was then formed which reported in 1901 (Fuller, 1902), in 1904 (Committee on Standard Methods of Water Analysis, 1905), and again in 1911 (Committee on Standard Methods for the Examination of Water and Sewage, 1912), recommending in considerable detail a standard routine procedure for the quantitative

and qualitative bacteriological examination of water for sanitary purposes. These reports have had a far-reaching effect in simplifying and unifying the methods of water analysis. Similar results have followed from the work of the English Committee appointed to consider the Standardization of Methods for the Bacterioscopic Examination of Water which reported in 1904, although this committee unfortunately did not consider the process of media making in great detail. The last report of the American Committee on Standard Methods (1912) will be adhered to in this and succeeding chapters unless otherwise specifically stated; and that portion of its report which deals with methods of making media will be found in full in the appendix.

Standard Procedure for Quantitative Determination of Bacteria in Water. The procedure for the quantitative determination of bacteria in water consists, in brief, in mixing a definite amount of a suitably collected specimen of the water with a sterile, solidifiable culture medium and incubating it for a sufficiently long time to permit reproduction of the bacteria and the formation of visible colonies which may be counted. The process is divided naturally into four stages—sampling, plating, incubating, and counting.

Sampling. All samples of water for bacteriological examination should be collected in clean, sterile bottles with wide mouths and glass stoppers, preferably of the flat mushroom type. It is desirable that these bottles should have a capacity of at least 100 c.c.

They should be cleaned thoroughly before using, by treatment with sulphuric acid and potassium bichromate

or with alkaline permanganate of potash followed by sulphuric acid, dried by draining, and sterilized by dry heat at 160° C. for at least 1 hour, or by steam at 115–120° for 15 minutes. If not to be used immediately the neck and stopper should be protected against dust or other contamination by wrapping with lead-foil. For transportation the bottle should be enclosed in a suitable case or box.

The greatest care must be taken that the fingers do not touch the inside of the neck of the bottle or the cone of the stopper, as the water thereby would become seriously contaminated and rendered unfit for examination. It is well known that bacteria are found abundantly upon the skin, and Winslow (Winslow, 1903) has shown that even B. coli is present upon the hands in a considerable number of cases.

In order to obtain a fair sample, great precautions must be taken, and these will vary with the different classes of waters to be examined and with local conditions. If a sample is to be taken from a tap, the water should be allowed to flow at least five minutes (if from a tap in regular use) or for a longer period in case the water has been standing in the house-service system. In the small pipes, changes in bacterial content are liable to occur, certain species dying and others multiplying.

If a sample is to be taken from a pump similar precautions are necessary. The pump should be in continuous operation for 5 minutes at least, and preferably for half an hour before the sample is taken, in order to avoid excessively high numbers due to the growth of

bacteria within the well and pump, the bacterial condition of the water as it passes through the ground being what we wish to determine. Thus Heræus (Heræus, 1886) in a well-water which had been but little used during the preceding 36 hours found 5000 organisms per c.c.; when the well was emptied by continuous pumping, a second sample, after an interval of half an hour, gave only 35. Maschek (Tiemann and Gärtner, 1889) obtained similar results, shown in the following table:

EFFECT OF PUMPING ON THE BACTERIAL CONTENT OF WELL-WATER

Well-water after continuous pumping for fifteen minutes	458
" " " " many hours	140
" later	68
" after continuous pumping for fifteen minutes	578
" " " many hours	179
" later	73

After a proper interval of pumping the sample of a well-water may be collected from the pet-cock of the pump or from a near-by tap. With a hand-pump, such as is found in domestic shallow wells, the water is, of course, pumped directly into the sample bottle. The difficulties in securing an average sample from this latter source are often great, since if the flooring about the pump is not tight, as is usually the case, continued pumping may wash in an unusual amount of surface pollution.

In sampling surface-waters, the greatest precautions must be observed to prevent contamination from the fingers. In still waters the fairest sample is one taken

from several inches down, as the surface itself is likely to have dust particles floating upon it. The method most frequently recommended is to plunge the bottle mouth downward to a depth of a foot or so, then invert and allow the bottle to fill.

Whenever any current exists, the mouth of the bottle should be directed against it in order to carry away any bacteria from the fingers. If there is no current, a similar effect can be produced by turning the bottle under water and giving it a quick forward motion. In rapidly flowing streams it is only necessary to hold the bottle at the surface with the mouth pointed up-stream.

For taking samples of water at greater depths, a number of devices have been employed, all of which are fairly satisfactory. The essentials are, first, a weight to carry the bottle down to the desired depth, and, second, some method of removing the stopper when that depth is reached. The student will find one good form of apparatus described in Abbott's "Principles of Bacteriology" (Abbott, 1899); an admirable one was devised by Hill and Ellms (Hill and Ellms, 1898); and Thresh (1904) figures an ingenious device for the same purpose. Miquel and Cambier (Miquel and Cambier, 1902) and other authors recommend the use of a sealed glass bulb with a capillary tube which can be broken off at the desired moment. Drew (1912) has devised an interesting sampling apparatus for use at great depths in the sea.

Changes in Bacterial Numbers after Sampling. As soon as a sample of water is collected its conditions

of equilibrium are upset and a change in the bacterial content begins. Even in the purest spring-waters, which contain but few bacteria when collected, and in which the amount of organic matter is infinitesimal, enormous numbers may be found after storage under laboratory conditions for a few days or even a few hours. In some cases the rise in numbers is gradual, in others very rapid. The Franklands (Frankland, 1894) record the case of a deep-well water in which the bacteria increased from 7 to 495,000 in 3 days. Miquel (Miquel, 1891) from his researches, arrived at the conclusion that in surface-waters the rise is less rapid than in waters from deep wells or springs, and that in the latter case the decrease, after reaching a maximum, is likewise rapid and steady. Just how far protection from light, increase in temperature, and a destruction of higher micro-organisms is responsible for the increase, and to what extent an exhaustion of food-supply or the formation of toxic waste products causes the succeeding decrease, we are not aware; but the facts are well established.

Whipple has exhaustively studied the details of this multiplication of bacteria in stored waters and has shown in the table given below that there is first a slight reduction in the number present, lasting perhaps for 6 hours; followed by the great increase noted by earlier observers. It is probable that there is a constant increase of the typical water bacilli, overbalanced at first by a reduction in other forms, for which the environment is unsuitable.

BACTERIAL CHANGES IN WATER DURING STORAGE

(WHIPPLE, 1901)

Sample	Initial Temperature.	Temp. of Incubation of Sample.	Number of Bacteria per c.c.				
			Initial.	After 3 Hours.	After 6 Hours.	After 24 Hours.	After 48 Hours.
	C.	C.					
A	7.6°	17.0°	260	215	230	900	27,000
B	7.6°	17.0°	260	245	255	720	10,850
C	7.6°	12.5°	260	270	231	600	2,790
D	7.6°	12.5°	260	270	245	710	1,800
E	7.6°	2.4°	260	243	210	675	1,980
F	7.6°	2.4°	260	235	270	560	1,980
G	11.0°	12.8°	77	55	58	101	10,250
H	11.0°	12.8°	77	53	74	87	2,175
I	11.0°	23.6°	77	51	52	11,000	41,400
J	6.7°	20.0°	430	375	245	.....	385,000[1]
K	6.7°	20.0°	430	345	405	.....	750,000[1]
L	23.2°	23.0°	510	340	230	8,000	20,000
M	23.2°	2.5°	525	300	220	380	2,200

[1] 0.0005 per cent peptone added to the water.

Wolffhügel and Riedel (Wolffhügel and Riedel, 1886) noted the dependence of this multiplication on the air-supply, vessels closed with rubber stoppers showing lower numbers than those plugged with cotton. Similarly, Whipple found that the multiplication of bacteria was much greater when bottles were only half full than when they were filled completely; and also, as shown in the very striking table on page 39, that the size of the bottle markedly influenced the growth.

An important series of investigations by Kohn (1906) suggests that this phenomenon of multiplication during storage may be due in part to the solution of certain constituents of glass which favor bacterial life, since the increase is notably greater in bottles of the more soluble glasses.

EFFECT OF SIZE OF VESSEL UPON THE MULTIPLICATION OF WATER BACTERIA DURING STORAGE

(WHIPPLE, 1901)

Sample	Bottle.	Temp. of Incubation.	Number of Bacteria per c.c.					
			Initial.[1]	After 3 Hrs.	After 6 Hrs.	After 12 Hrs	After 24 Hrs.	After 48 Hrs.
		C						
A	1-gallon	13°	77	63	65	47	42	175
B	2-quart	13°	77	59	63	60	45	690
C	1-quart	13°	77	63	63	47	46	325
D	1-pint	13°	77	57	61	36	38	630
E	2-ounce	13°	77	55	58	47	101	10,250
F	1-gallon	24°	77	81	97	275	290	300
G	2-quart	24°	77	92	59	62	180	250
H	1-quart	24°	77	84	77	46	340	900
I	1-pint	24°	77	51	46	100	2,950	7,020
J	2-ounce	24°	77	51	52	145	11,000	41,400

[1] Average of five plates.

Whipple's table, quoted above, shows that the multiplication during storage was greater at a higher temperature; and this is a well-recognized general rule. In order to obviate the abnormal results of storage increase it is therefore obvious that samples must be examined shortly after collection and that they must be kept cool during their necessary storage. If fairly pure waters are placed upon ice and kept between 0 degrees and 10 degrees, they will show no material increase in 12 hours. With polluted water, however, another danger is here introduced. Samples of such water when packed in ice show a marked decrease due to the large number of sensitive intestinal bacteria present. Jordan (Jordan, 1900) found that three samples of river-water packed in ice for 48 hours fell

off from 535,000 to 54,500; from 412,000 to 50,500, and from 329,000 to 73,000, respectively. It is, therefore, important that even iced samples should not be kept too long; and it is desirable to adhere strictly to the recommendations of the Standard Methods Committee that the interval between sampling and examination should not exceed 12 hours in the case of relatively pure waters, 6 hours in the case of relatively impure waters, and 1 hour in the case of sewage.

Plating. The bottle containing the sample of water is first shaken at least twenty-five times in order to get an equal distribution of the bacteria. If the number of bacteria present is probably not greater than 200, 1 c.c. is then withdrawn with a sterile 1 c.c. pipette and delivered into a sterile Petri dish of 10 cm. diameter. To this is added 5 c.c. of standard 10 per cent gelatin at a temperature of about 30° C., or standard agar (7 c.c.) at 40–42° C. Should the number of bacteria per c.c. probably exceed 200, dilution is necessary. This is best accomplished by adding 1 c.c. of the water in question to 9, 99 or 999, etc., c.c. of sterile tap water according to the amount of dilution required. The diluted sample is then shaken thoroughly and 1 c.c. taken for enumeration. In order to determine the number of bacteria originally present it is only necessary to multiply by the factor 10, 100, or 1000, etc.

When a sample of water from an unknown source is to be examined it is generally desirable to make two check plates at each of the above dilutions, selecting those which give nearest to 200 colonies on the plates after incubation as the ones on which to rely

for the count. A much smaller number will not give average figures, and if more than 200 colonies are present on a plate many bacteria will be checked by the waste products of those which first develop and the count obtained will be too low. After the addition of the diluted sample and the nutrient medium, their thorough mixture in an even layer on the bottom of the plate is obtained by careful tipping and rotation.

It was formerly customary to mix the water with the gelatin in the tube before pouring into the plate, but this method is objectionable because there is always a residuum of medium remaining in the tube which will retain varying numbers of bacteria and thus interfere with the accuracy of the count. Before pouring the medium into the plate the mouth of the tube should be flamed to remove any possibility of contamination.

The usual method of determining the number of bacteria in water for sanitary purposes in Germany, England and the United States has always been by the use of gelatin plates with a 2-day incubation period at 20 degrees. The 1905 Standard Methods Report of the American Public Health Association Committee recommended this procedure, which has been universally adopted. The 1912 Report, however, suggests the use of agar with a 1-day period at 37 degrees, as yielding quicker results and indicating the presence of bacteria more nearly related to pathogenic types. The comparative value of the two methods has been well discussed by Whipple (1913). The use of gelatin is not only more time-consuming, but requires the use of a

special 20-degree incubator which is difficult to regulate. The 37-degree incubator must be provided in any case for the isolation of B. coli. On the other hand, the time seems hardly ripe for the abandonment of the 20-degree count, which has been used for 20 years all over the civilized world, and for the interpretation of which we have very complete data. There is at present no such sound basis for interpreting the 37-degree count, and in many cases, as in the control of water filtration plants, the 37-degree numbers are too small to be of any practical value. Furthermore the 20-degree count may furnish evidence of surface contamination as distinguished from fecal pollution, which is often of considerable value.

The authors have always urged the use of the 37-degree count along with the 20-degree count as furnishing most valuable information; but this is very different from the substitution of one count for the other. The recommendation that the 20-degree count be abandoned, with no evidence to warrant such a revolutionary change, and no experimental results on which to base an interpretation of the 37-degree count, has aroused vigorous opposition from a large majority of practical water bacteriologists. At the Washington meeting of the American Public Health Association in September, 1912, it was resolved " that in the opinion of the Laboratory Section of the American Public Health Association, ordinary routine examination of water for sanitary purposes, and in the control of purification plants, for the present should include the determination of the number of bacteria developing

at 20 degrees and at 37 degrees and a presumptive test for B. coli in lactose bile."

This action of the section responsible for the appointment of the Standard Methods Committee appears to supersede the report of the committee itself and makes the combination of the 20- and 37-degree counts the standard American procedure. The 20-degree count may be made on either gelatin or agar; but it is the 20-degree count which will be discussed in this chapter, leaving the body temperature count for consideration in Chapter IV.

The exact composition of the medium is, of course, of prime importance in controlling the number of bacteria which will develop. The figures previously cited in connection with the discussion of Hesse's Nährstoff agar show how bacterial counts may vary with media of widely different composition. The table quoted on page 44 from Gage and Phelps (1902), shows the considerable differences which may be due to the presence or absence of meat infusion, peptone, etc., in media of generally similar character (compare the figures for plain gelatin, peptone, gelatin, and meat gelatin). Much slighter variations than this, however, are significant. The reaction of the medium was found as early as 1891 to be important, for Reinsch (Reinsch, 1891) showed in that year that the addition of one one-hundredth of a gram of sodium carbonate to the liter increased sixfold the number of bacteria developing. Fuller (Fuller, 1895) and Sedgwick and one of us (Sedgwick and Prescott, 1895), working independently, established the fact that an optimum reaction

existed for most water bacteria and that a deviation either way decreased the number of colonies developing.

TABLE SHOWING PERCENTAGES OF BACTERIA DEVELOPING ON MEDIA OF DIFFERENT COMPOSITIONS

(Gage and Phelps, 1902)

Medium.	Days' Count.							
	2	3	4	5	6	7	8	9
Nährstoff agar	19	60	78	85	95	99	99	100
Nährstoff peptone agar	10	22	26	28	30	30	30	30
Peptone agar	11	16	22	23	24	24	24	24
Meat agar	8	13	16	17	17	17	17	17
Plain agar	8	10	13	14	14	14	14	14
Regular agar	7	9	11	11	11	11	11	11
Nährstoff glycerin agar	6	10	11	11	11	11	11	11
Nährstoff meat agar	7	7	8	8	10	10	10	10
Meat gelatin	12	19	24	26	26	26	26	26
Peptone gelatine	7	12	18	20	20	20	20	20
Standard gelatin	8	10	11	12	13	13	13	13
Plain gelatin	1	6	12	13	13	13	13	13
Nährstoff gelatin	5	6	9	11	13	13	13	13

Whipple (Whipple, 1902) has shown that not only the particular kind of gelatin used, but its exact physical condition as affected by sterilization and other previous treatments, will materially affect the results obtained. Gage and Adams (1904) found marked differences in counts as the result of the use of the two best-known commercial peptones. A long series of waters plated on agar made up with Merck's and Witte's peptones, respectively, showed the average relative results in the table on page 45.

AVERAGE RELATIVE NUMBER OF BACTERIA ON PEPTONE AGAR WITH DIFFERENT PEPTONES

(GAGE AND ADAMS, 1904)

DAYS	2	4	6	8	10	12
Merck's.....	9	33	51	67	89	98
Witte's.....	38	53	100	100	100	100

The same authors showed that the composition of the water used exercised a marked selective action upon the development of bacteria. Agar made up with sewage permitted a maximum growth of sewage bacteria and showed no colonies when inoculated with filtered city water. On the other hand agar made up with city water showed 100 per cent of the bacteria present in city water and river water, three-quarters of those present in sewage and less than half of those present in sewage effluents.

Hesse (1904) found that the number of bacteria developing on Nährstoff agar varied with the composition of the glass tubes in which the media had previously been sterilized. The more soluble glasses yielded sufficient alkali to the medium to inhibit four-fifths of the bacteria present in certain cases.

All these facts make it evident that only the strictest adherence to a standard method can ensure comparable results; the ordinary nutrient gelatin or agar should then in all practical sanitary work be made up from distilled water, meat infusion, peptone and gelatin or agar, in exact accordance with the directions of the Standard Methods Committee.

tilated dishes and by the presence of a pan of water in the incubating chamber.

According to American and German practice, plates made for sanitary water analysis are counted at the end of 48 hours. The English Committee appointed to consider the standardization of methods for the Bacterioscopic Examination of Water (1904) fixed the time at 72 hours. French bacteriologists, and some Germans (Hesse and Niedner, 1906), still recommend longer periods, and the following table from Miquel and Cambier (Miquel and Cambier, 1902) shows that many bacteria fail to appear in our ordinary procedure. It is, however, in the main, the characteristic water bacteria which develop slowly, sewage bacteria almost without exception being rapid growers. The longer period of incubation is, therefore, not only inconvenient, but undesirable, since it obscures the difference between good and bad waters.

EFFECT OF THE LENGTH OF INCUBATION OF WATER BACTERIA IN GELATIN UPON THE NUMBER OF COLONIES DEVELOPING

(Miquel and Cambier, 1902)

Length of Incubation.	Colonies Developed.	Length of Incubation.	Colonies Developed.
1 day	20	9 days	821
2 days	136	10 days	859
3 days	254	11 days	892
4 days	387	12 days	921
5 days	530	13 days	951
6 days	637	14 days	976
7 days	725	15 days	1000
8 days	780		

Counting. The number of bacteria is determined by counting the colonies developed upon the plate, with the aid of a lens magnifying at least five diameters. For convenience in counting the plate may be placed upon a glass plate ruled in centimeter squares and set over a black tile; or the tile itself may be ruled. As has already been said, it is desirable that the number of colonies should not exceed 200, for when the number is very high the colonies grow only to a small size, making counting laborious and inaccurate, and many do not develop at all. The best results are obtained with numbers ranging from 50 to 200.

When it is possible to do so, all the colonies on the plate should be counted. When they exceed 400 or 500 it is often easier, and fully as accurate, to count a fractional part of the plate and estimate the total number therefrom. This should not be done, however, except in case of necessity.

Ayers (1911) has suggested two counting devices which will be found very useful where a great many plates have to be handled. For getting the best possible transmitted light, he places his plate on the ground-glass top of a wooden box, 7 inches square, with one side open to admit light, which is reflected upward by a plane mirror set in the box at an angle of 45 degrees. An ordinary graduated-glass counting plate may be placed between the ground-glass and the Petri dish, and the eyes are protected from direct light by a screen rising from the open side of the box. For picking colonies from a gelatin plate in a warm room, he places between the ground glass and the Petri dish a copper

box with top and bottom of glass 7 inches square and 1½ inches deep, through which cold water is allowed to circulate.

Expression of Quantitative Results. It is customary in determining numbers to make plates in duplicate, thereby affording a check upon one's own work. Owing to the lack of precision in the method, the limit of experimental error is a wide one. It should be possible for careful manipulators to obtain results within 10 per cent of each other, but a closer agreement than this is hardly to be expected. It has been suggested by the committee of the American Public Health Association that the following mode of expressing results be adopted in order to avoid the appearance of a degree of accuracy which the methods do not warrant.

NUMBERS OF BACTERIA FROM

1–50	shall be recorded to the nearest unit			
51–100	"	"	"	5
101–250	"	"	"	10
251–500	"	"	"	25
501–1000	"	"	"	50
1001–10,000	"	"	"	100
10,001–50,000	"	"	"	500
50,001–100,000	"	"	"	1,000
100,001–500,000	"	"	"	10,000
500,001–1,000,000	"	"	"	50,000
1,000,001–5,000,000	"	"	"	100,000

The determination of numbers of bacteria in water in the field has frequently been attempted. Since the laboratory method of "plating out" is difficult to use in field work, the Esmarch tube process has often been employed. This consists in introducing into a tube of melted gelatin or agar 1 c.c. of the water and then

rotating the tube until the medium has solidified in a thin layer on the inner wall. Other bacteriologists have devised ingenious field kits for adapting the plate method to this purpose, of which one very good form has recently been described by Van Buskirk (1912). The opportunity for air infection in work done outside a proper laboratory is, however, always great; and it is almost impossible to secure proper conditions for incubation in any makeshift establishment. On the whole, the authors are of the opinion that laboratory examinations are to be preferred to those made in the field, if a laboratory can be reached within 12 hours or so of the time of collection of the samples.

The Kansas State Water Survey has made use of a device for shipping iced samples which has given excellent results. The outfit is described in the American Journal of Public Health for May, 1914, and consists essentially of an insulated container in which the bottles are firmly held by a simple mechanism. With proper icing the samples are maintained at a temperature below 10 degrees C. for two or three days or even more. Very little change due to storage is found in the counts on agar when plates are incubated at 37 degrees C. and in winter the 20-degree counts are satisfactory on agar. Gelatine counts were found to be erratic and not dependable.

The following tables kindly sent us by Prof. C. C. Young show the comparative numbers obtained in the laboratory after certain periods of storage, and in the laboratory as compared with the actual field analyses.

EFFECT OF STORAGE ON BACTERIAL CONTENT

Place Collected.	Total Count Agar, 37 Deg. C., 24 hrs.	Total Count Gelatine 20 Deg. C., 48 hrs.	B. coli per c.c.		
			Lactose Bile	Lactose Broth	Dextrose Bile
Time—immediately					
River	600	3,000	0.4	6.0	6.0
Settling basin	36	250	0	0.2	0.2
Tap at 1323 Ky.	11	1,500	0	0	0
Blackmar Well	18	5	0	0	0
Well at 1338 Ohio	7	35	0.2	0	0
Time—12 hours					
River	820	28,000	0.1	2.0	2.0
Settling basin	50	2,000	0.1	0.2	0.2
Tap at 1323 Ky.	25	500	0	0	0
Blackmar Well	30	250	0	0	0
Well at 1338 Ohio	10	80	0.1	0.1	0
Time—48 hours					
River	600	55,000	0.1	0.4	0.4
Settling basin	20	1,800	0	0.2	0.2
Tap at 1323 Ky.	40	10,000	0.1	0.1	0
Blackmar Well	12	350	0	0	0
Well at 1338 Ohio	10	50	0.1	0.2	0.2
Time—immediately					
River	750	6,200	8.0	6.0	6.0
Laboratory tap	10	100	0	0	0
Blackmar Well	8	300	0	0	0
Beamer Well	35	500	4.0	4.0	2.0
Time—12 hours					
River	850	60,000	4.0	6.0	4.0
Laboratory tap	15	25	0	0	0
Blackmar Well	4	1,500	0	0	0
Beamer Well	40	1,000	4.0	4.0	4.0
Time—24 hours					
River	920	2,900	6.0	40.0	8.0
Laboratory tap	20	1,400	0	0	0
Blackmar Well	200	1,500	0	0	0
Beamer Well	100	500	0.8	4.0	1.0

EFFECT OF STORAGE ON BACTERIAL CONTENT
(*Concluded*)

Place Collected.	Total Count Agar, 37 Deg. C. 24 hrs.	Total Count Gelatine 20 Deg.C. 48 hrs.	B. coli per c.c.		
			Lactose Bile.	Lactose Broth.	Dextrose Bile.
Time—48 hours					
River	800	5,500	20.0	20.0	20.0
Laboratory tap	12	120	0	0	0
Blackmar Well	150	2,000	0	0	0
Beamer Well	120	800	0.8	2.0	1.0
Time—immediately					
River	2500	.....	100.0	100.0	100.0
Tap at laboratory	3200	.....	0.8	0.8	0.8
Blackmar Well	175	.....	1.0	1.0	1.0
Public Well, Mass. st.	230	.....	10.0	10.0	10.0
Time—24 hours					
River	4000	.....	100.0	100.0	100.0
Tap at laboratory	1900	.....	0.4	0.6	0.2
Blackmar Well	300	.....	0.6	0.6	0.4
Public well on Mass. st.	320	.....	10.0	4.0	2.0
Time—48 hours					
River	2200	.....	100.0	100.0	100.0
Tap at laboratory	1400	.....	0.6	0.6	0.4
Blackmar Well	400	.....	0.4	0.6	0.2
Public well on Mass. st.	1200	.....	1.0	1.0	1.0

CHAPTER III

THE INTERPRETATION OF THE QUANTITATIVE BACTERIOLOGICAL EXAMINATION

Standards for Potable Water. The information furnished by quantitative bacteriology as to the antecedents of a water is in the nature of circumstantial evidence and requires judicial interpretation. No absolute standards of purity can be established which shall rigidly separate the good from the bad. In this respect the terms "test" and "analysis" so universally used are in a sense inappropriate. Some scientific problems are so simple that they can be definitely settled by a test. The tensile strength of a given steel bar, for example, is a property which can be determined. In sanitary water examination, however, the factors involved are so complex, and the evidence necessarily so indirect, that the process of reasoning much more resembles a doctor's diagnosis than an engineering test.

The older experimenters attempted to establish arbitrary standards, by which the sanitary quality of a water could be fixed automatically by the number of germs alone. Thus Miquel (Miquel, 1891) published a table according to which water with less than 10 bacteria per c.c. was "excessively pure," with 10 to 100

bacteria, "very pure," with 100 to 1000 bacteria, "pure," with 1000 to 10,000 bacteria, "mediocre," with 10,000 to 100,000 bacteria, "impure," and with over 100,000 bacteria, "very impure." Few sanitarians would care to dispute the appropriateness of the titles applied to waters of the last two classes; but many bacteriologists have placed the standard of "purity" much higher. The limits set by various German observers range, for example, from 50 to 300. Dr. Sternberg (Sternberg, 1892) in a more conservative fashion, has stated that a water containing less than 100 bacteria is presumably from a deep source and uncontaminated by surface drainage; that one with 500 bacteria is open to suspicion; and that one with over 1000 bacteria is presumably contaminated by sewage or surface drainage. This is probably as satisfactory an arbitrary standard as could be devised, but any such standard must be applied with great caution. The source of the sample is of vital importance in the interpretation of analyses; a bacterial count which would condemn a spring might be quite normal for a river; only figures in excess of those common to unpolluted waters of the same character give an indication of danger. Furthermore, the bacteriological tests are far more delicate than any others at our command, very minute additions of food material causing an immense multiplication of the microscopic flora. This delicacy necessarily requires, both in the process of analysis and the interpretation of results, a high degree of caution. As pointed out in the previous chapter, the touch of a finger or the entrance of a particle of dust may wholly

destroy the accuracy of an examination. Even the slight disturbance of conditions incident upon the storage of a sample after it has been taken may in a few hours wholly alter the relations of the contained microbic life. It is necessary, then, in the first place, to exercise the greatest care in allowing for possible error in the collection and handling of bacteriological samples; and in the second place, only well-marked differences in numbers should be considered significant.

In the early days of the science, discussion ran high as to the interpretation of bacteriological analysis; and particularly as to the relation of bacterial numbers to the organic matter present in a water. Different observers obtained inconsistent results, and Bolton (Bolton, 1886) concluded that there was no relation whatever between the organic pollution of a water and its bacterial content. Tiemann and Gärtner (Tiemann and Gärtner, 1889) furnished the key to the difficulty in their statement that there are two classes of bacteria, the great majority of species normally occurring in the earth or in decomposing organic matter, which require abundance of nutriment, and certain peculiar water bacteria which can multiply in the presence of such minute traces of ammonia as are present in ordinary distilled water. Even these prototrophic or semi-prototrophic forms, however, require a definite amount of food of their own kind.

Kohn (1906) determined the minimal nutrient material requisite for certain of them and found that they could develop in the presence of 198×10^{-10} to 198×10^{-13} per cent of dextrose, 66×10^{-13} to 66×10^{-17} per cent

ammonium sulphate and 66×10^{-13} to 66×10^{-19} per cent ammonium phosphate. Similar minute amounts of organic matter are found in the purest of natural waters and under exceptional conditions certain species of bacteria may therefore multiply in bottled samples, or, at times, in a well or the basin of a spring. In normal surface-waters, such growths of the prototrophic forms do not apparently occur. Here it is found as a matter of practical experience that the number of bacteria present depends upon the extent to which the water has been contaminated with decomposing organic matter, either by pollution with sewage or by contact with the surface of the ground. The bacterial content varies as the extent and character of the contamination varies. It measures not merely organic matter, but organic matter in a state of active decay, and like the ammonias and other features of the sanitary chemical analysis, indicates fresh organic pollution, with the added advantage that the presence of the stable nitrogenous compounds often present in peaty waters introduces no error in the bacteriological analysis.

Bacterial Content of Surface-waters. In judging of a surface-water the student will be aided by reference to the figures given for certain normal sources in Chapter I; the Boston tap water with 50 to 200 bacteria per c.c. (Philbrick, 1905) and the water of Lake Zurich with an average of 71 in summer and 184 in winter (Cramer, 1885) may be taken as typical of good potable waters; and numbers much higher than these are open to suspicion, since all contamination whether contributed by sewage or by washings from the surface of the

ground is a possible source of danger. The excess of bacteria in surface-waters during the spring and winter months is by no means an exception to the general rule that high numbers are significant, since the peril from supplies of this character is clearly shown by the spring epidemics of typhoid fever which at the times of melting snow visit communities making use of unprotected surface-waters. Streams receiving direct contributions of sewage exhibit a similar excess of bacteria at all times, numbers rising to an extraordinary height near the point of pollution and falling off below as the stream suffers dilution and the sewage organisms perish. Miquel (Miquel, 1886) records 300 bacteria per c.c. in the water of the Seine at Choisy, above Paris; 1200 at Bercy in the vicinity of the city, and 200,000 at St. Denis after the entrance of the drainage of Paris. Prausnitz (Prausnitz, 1890) found 531 bacteria per c.c. in the Isar above Munich, 227,369 near the entrance of the principal sewer, 9111 at a place 13 kilometers below the city, and 2378 at Freising, 20 kilometers further down. Jordan (Jordan, 1900), in his study of the fate of the sewage of Chicago, found 1,245,000 bacteria per c.c. in the drainage canal at Bridgeport, 650,000 at Lockport, 29 miles below, and numbers steadily decreasing to 3660 at Averyville, 159 miles below the point of original pollution. Below Averyville the sewage of Peoria enters and the numbers rise to 758,000 at Wesley City, decreasing to 4800 in 123 miles flow to Kampsville. Brezina (1906) found 1900 bacteria per c.c. in the Danube River above, and 110,000 at the north of the Danube canal. This number

fell to 85,000 one kilometer below, 62,000 four kilometers below, and 40,000 seven kilometers down the stream. Vincent (1905) records from 1000 to 46,000 bacteria per c.c. in the waters of more or less polluted French rivers. Mayer (1902), on the other side of the world, found 21 and 35 bacteria per c.c. in the Shaho River, near its source, in the vicinity of the great Chinese Wall and from 100,000 to 600,000 in the highly polluted Whangpo near its mouth.

Bacterial Content of Ground-waters. In ground-waters we have seen that bacteria may occasionally be present in considerable numbers, but if so they are generally organisms of a peculiar character, incapable of development on the ordinary nutrient media in the standard time. Thus in 48 hours we often obtain counts measured only in units or tens such as have been recorded in Chapter I. When higher numbers are present, the general character of the colonies must be taken into account, since besides the slowly-growing forms certain other water bacteria, which require a comparatively small amount of nutriment, may multiply at times in a deep well or the basin of a spring. In such a case, however, the appearance of the plates at once reveals the peculiar conditions, for the colonies are of one kind and that distinct from any of the sewage species. Thus Dunham (Dunham, 1889) reports that the mixed water from a series of driven wells gave 2 bacteria per c.c., while another well, situated just like the others, contained 5000, all belonging to a single species common in the air. Except in such peculiar cases as this high numbers in a ground-water mean contamination.

Bacteria in Filtered Waters. The process of slow sand filtration for the purification of unprotected surface-water is essentially similar to the action which takes place in nature when rain soaks through the ground to appear in wells and springs; and it is in the examination of the effluent from such municipal plants that the quantitative bacteriological analysis finds, perhaps, its most important application. The chemical changes which occur in the passage of water through sand at a rate of 1,000,000 or 2,000,000 gallons per acre per day are so slight as to be negligible. The bacteria present should, however, suffer a reduction of 98 or 99 per cent, and their numbers furnish the best standard for measuring the efficiency of such filtration plants. At Lawrence, in 1905, Clark found an average of 12,700 bacteria per c.c. in the raw water of the Merrimac River, while the number present in the filtered water was only 70 (Massachusetts State Board of Health, 1906). Where the number of bacteria in the applied water is smaller it is difficult to obtain so high a percentage efficiency. At Washington, for example, prolonged sedimentation generally reduces the bacterial numbers to less than a thousand and it is almost impossible to secure a 99 per cent removal. The actual numbers of bacteria in the effluent are, however, much lower than at Lawrence. The monthly average results obtained for a year at these two plants are tabulated on page 58.

Mechanical filtration gives similar results. Fuller at Cincinnati (Fuller, 1899) records 27,200 organisms per c.c. in the water of the Ohio River between

September 21, 1898, and January 25, 1899, while the average content of the effluent from the Jewell filter was 400. Data with regard to the operation of mechanical filters are now abundant, since all over the world the operation of these plants is controlled by bacteriological methods. Recently Johnson (1907) has reported some interesting results from the far East. At Osaka, Japan, an average of 200 bacteria per c.c. in the raw water of the Yodo River was reduced, in 1905, to an average of 25 by slow sand filters; at Bethmangala, India, in 1906, mechanical filters treated the water of the Palar River, containing 4350 bacteria per c.c., and yielded an effluent with only 13 per c.c. (Johnson, 1907).

The average monthly results obtained with the new mechanical filter plant at Harrisburg, Pa., are included in the table below for comparison with the figures

REMOVAL OF BACTERIA BY NATURAL SAND FILTERS AND MECHANICAL FILTERS. BACTERIA PER C.C. IN APPLIED WATER AND EFFLUENT. MONTHLY AVERAGES

Month.	Washington, 1906.		Lawrence, 1905.		Harrisburg, 1906.	
	Applied Water.	Effluent.	Applied Water.	Effluent.	Applied Water.	Effluent.
January	1500	39	14,200	110	9,510	104
February ...	550	16	14,800	55	21,228	298
March.....	650	19	10,300	55	31,326	75
April.....	400	22	3,600	170	39,905	42
May.....	65	17	1,900	12	6,187	86
June.....	220	17	9,600	9	2,903	31
July.....	160	26	3,900	55	685	10
August	190	14	19,500	37	1,637	5
September ..	130	14	13,500	44	836	12
October.....	275	16	39,800	110	7,575	63
November ..	220	12	8,700	70	26,224	236
December....	700	45	..	..	37,525	163

recorded at Washington and Lawrence; and these may be taken as typical, since the Harrisburg plant is the latest of its type, as the Washington plant is the newest and most perfectly equipped of slow sand filters.

In well-managed purification plants the bacteria in the effluent are determined daily, and any deviation from the normal value at once reveals disturbing factors which may impair the efficiency of the process. In Prussia official regulations demand such systematic examinations and prescribe 50 as the maximum number of bacteria allowable in the filtered water. In the same way the condition of an unpurified surface supply may be determined by daily bacteriological analyses and warnings of danger issued to the public, as has been done at Chicago and other cities. In general, any regular determination of variations from a normal standard furnishes ideal conditions for the bacteriological methods; and the detection by Shuttleworth (Shuttleworth, 1895) of a break in a conduit under Lake Ontario by a rise in the bacteria of the Toronto water-supply may be cited as a classic example of its application.

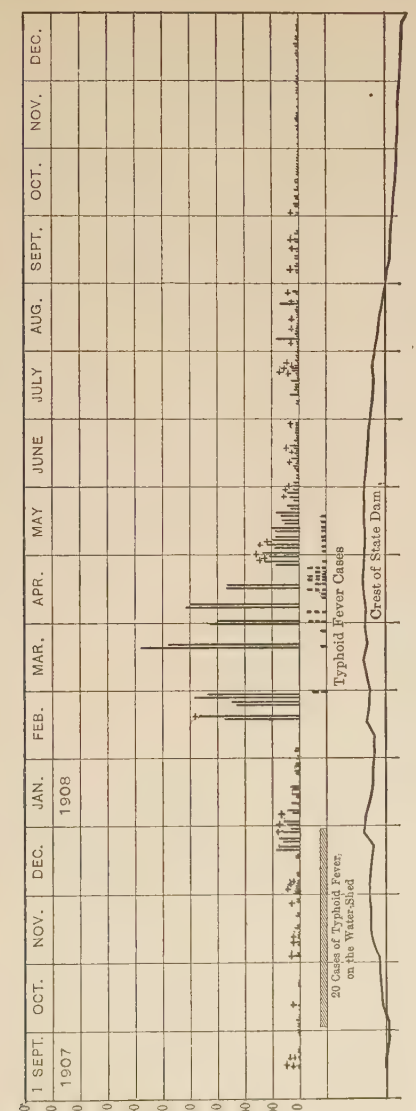
Often, however, the expert is called to pass upon the character of a water of which no series of analyses is available. In such cases an inspection of the location from which the water comes should be insisted on, as a sound interpretation of a water analysis can only be made with a reasonably full knowledge of the source of the sample. After a careful sanitary inspection, however, the comparison of the result of even a single examination with the normal range for waters of the same class

may prove of great significance, as a few practical examples may make clear (Winslow, 1901).

In the spring of 1900 the city of Hartford, Conn., was using a double supply, from the Connecticut River and from a series of impounding reservoirs among the hills. A single series of plates showed from 4000 to 7000 bacteria per c.c. in the water of the river, while the reservoir water contained 300 to 900. The abandonment of the river supply followed, and at once the excessive amount of typhoid fever in the city was curtailed.

In the fall of 1900, Newport, R. I., experienced an outbreak of typhoid fever, and when suspicion was thrown upon the surface water-supply, chemical analysis of the latter was not wholly reassuring; but there were only 334 bacteria per c.c. in the water from the taps, while a well in the infected district gave 6100. It was no surprise to find, on a further study of the epidemic, that the well was largely at fault and the public supply was not.

In the case of ground-water the evidence is usually even more distinct. At Framingham, Mass., in 1903, high chlorin content in the public supply, drawn from a filter gallery beside a lake, had led to public anxiety. Five samples from different parts of the system showed averages of 1, 2, 2, 2, and 4 bacteria per c.c.; and taking this in conjunction with the other features of the bacteriological analysis, it was possible to report that any pollution introduced upon the gathering ground had at the time of examination been entirely removed.



Changes in Total Count and B. coli Content in Lake Owasco During the Year of the Water Epidemic of Typhoid Fever at Auburn, N. Y.

(Courtesy of J. Walter Ackerman.)

The results presented in the diagram on page 60*a* offer an interesting example of the value of the total count of bacteria as compared with the estimation of B. coli. This chart, prepared by Mr. J. W. Ackerman, the Engineer of the Auburn, N. Y., Water Board, shows by the vertical lines the total count and by the crosses the presence of colon bacilli in the water supply of Auburn drawn from Lake Owasco. In the year illustrated in this diagram, and in other years, before and after, the total count of bacteria rose sharply at the time of the spring thaw, while colon bacilli were on the whole more abundant during the summer. The explanation of this phenomenon probably is that a certain proportion of colon bacilli are always contributed by the small brooks which enter the reservoir from agricultural land which, not being of human origin, have but little significance. At the time of the spring thaws, which for the most part wash an open farming country, the normal contribution of B. coli from the fields is obscured by the rain and melting snow, while only the rise in total count registers the fact that contaminating material of all sorts is being washed into the lake. With this contaminating material, for the most part of a harmless nature, human excreta are washed in from certain points on the watershed, and in the spring of 1908 these excreta contained specific typhoid infection and an epidemic in Auburn was the result.

In this case then the total count of bacteria was a more accurate index of danger than the colon content.

CHAPTER IV

DETERMINATION OF THE NUMBER OF ORGANISMS DEVELOPING AT THE BODY TEMPERATURE

Relation between Counts Made at 20° and 37°. The count of colonies upon the gelatin plate measures, as we have pointed out, the number of the metatrophic bacteria in general; and the distribution of these forms corresponds with the decomposition of organic matter wherever it may occur. In this great class, there are some species which will grow under a wide variety of conditions. These are present in most waters in small numbers, and in sources contaminated with wash from decaying vegetable matter they occur in abundance. Other metatrophic forms, however, through a semi-parasitic mode of life, have become specially adapted to the peculiar conditions characteristic of the animal body; and these bacteria possess the property of developing most actively at the temperature of the human body, 37° C., which altogether checks the growth of the majority of normal earth and water forms. The determination of the number of organisms growing at the body temperature may throw light, then, on the presence of direct sewage pollution, since the bacteria from the alimentary canal flourish under such conditions, while most of those derived from other sources do not. Savage classifies the bacteria which may

be found in water under three headings: normal inhabitants, like B. fluorescens; unobjectionable aliens (from soil), like B. mycoides, and objectionable aliens (from excreta), like B. coli. The first sort and many of the second sort are generally unable to grow at 37 degrees. This criterion is not an absolute one. Savage, (1906) reports an experiment in which unpolluted soil, which had not been manured or cultivated for at least 3 years, was added to tap water, with the result that a 20° count of 76 was increased to 1970, and a 37° count of 3 was raised to 1630. In this case most of the bacteria in the soil were capable of development at body temperature. Experience shows, however, that the numbers of such bacteria which actually reach natural waters from such sources are seldom large. The count at 37°, therefore, helps to distinguish contamination by wash of the soil of a virgin woodland from pollution by excreta, since in the former case the proportion of blood-temperature organisms is much smaller than in the latter. Furthermore, this method is free from much of the error introduced by the multiplication of bacteria after the collection of a sample, as most of the forms which grow in water during storage cannot endure the higher temperature and consequently do not develop upon incubation. Recently, for example, water from a spring of good quality was shipped to the laboratory from a considerable distance. Gelatin plates showed 4200 bacteria per c.c., but agar plates at 37° were sterile.

Significance of the 37° Count. A majority of the English Committee appointed to consider the standard-

ization of methods for water examination (1904) recommended the body-temperature count as a standard procedure. The American Committee on Standard Methods in its 1905 Report did not recommend this method even for alternative use. In its last report (1912), however, it substituted the 37° for the 20° count, which was dropped out entirely. As we have pointed out in Chapter II, this course seems to us an unwise one, and it was formally condemned at the meeting of the Laboratory Section of the American Public Health Association in September, 1912, by the passage of a vote declaring that "ordinary routine examinations of water for sanitary purposes, and in the control of purification plants for the present, should include the determination of the number of bacteria developing at 20 degrees and 37 degrees." By this action the body-temperature count is placed on a par with the 20-degree count as an integral part of sanitary bacteriological water examination, a course which has been strongly urged in earlier editions of this book.

The body-temperature count must, of course, be made upon agar plates; but otherwise the procedure is much the same as that already described for the routine quantitative bacteriological examination in Chapter II. A 1.5 per cent agar medium has generally been used, but the Standard Methods Committee in its recent report recommends only 1 per cent of agar. Whipple (1913) points out that this 1 per cent agar often gives trouble from the running together of the colonies on the weaker medium. On the other hand, a 1 per cent agar gives higher counts than 1.5 per cent

agar. He emphasizes the recommendation of Jackson that the agar used should be dried at 105° C. for 30 minutes, as commercial agar itself contains more or less water.

The period of incubation ordinarily adopted for body-temperature counts is 24 hours. Lederer and Bachmann (1911) find that with sewage effluents a 48-hour period at 37° may yield counts from two to six times as high as those obtained in 24 hours; it is questionable, however, whether the higher counts thus given would compensate for the loss of time. The adoption of a 24-hour period by the Standard Methods Committee in any case represents an almost universal practice.

In using agar plates at 37° difficulty is sometimes caused by the spreading of colonies of certain organisms over the surface of the plate in the water of condensation which gathers; this may be avoided by inverting the plates after the agar is once well set, or still better by the use of plates provided with earthenware tops, as suggested by Hill. The porous earthenware absorbs the water which condenses on it, the surface of the plate remains comparatively dry, and the percentage of "spread" plates is reduced from 30 per cent to 1 per cent (Hill, 1904). Special pains must be taken, however, to keep the atmosphere in the incubator nearly saturated with moisture or errors will be introduced by the excessive evaporation of the medium used.

Use of Litmus Lactose Agar. Additional evidence as to the character of a water sample may be

obtained with little extra trouble by adding a sugar and some sterile litmus to the agar medium and observing the fermenting powers of the organisms present, as first suggested by Wurtz (Wurtz, 1892) for the separation of B. coli from B. typhi. It happens that the most abundant intestinal organisms, belonging to the groups of the colon bacilli and the streptococci, decompose dextrose and lactose with the formation of a large excess of acid. The decomposition of the latter sugar, is, on the other hand, almost entirely wanting among the commoner saprophytic bacteria, and therefore lactose is most commonly used in making sugar agar, 1 per cent being added to the medium just before the final filtration (between steps 15 and 16 in the standard process of media-making given on p. 271). In pouring the plate a cubic centimeter of sterile litmus solution should be added. After incubation the colonies of the acid-forming organisms will be clearly picked out by the reddening of the adjacent agar. Only those colonies which are sharply colored should be considered as significant, since certain bacteria of the hay-bacillus group produce weak acid and faint coloring of the litmus.

When polluted waters are examined in this manner the number of organisms developing on the lactose-agar plate will be very high, almost equalling in some cases the total count obtained on gelatin. Chick (Chick, 1901), using a lactose-agar medium with the addition of one-thousandth part of phenol, found, of colon bacilli alone, 6100 per c.c. in the Manchester ship canal; 55–190 in the polluted River Severn, and

numbers up to 65,000 per gram in roadside mud. In an examination of water from the Charles River above Boston, 37° counts ranging from 9800 to 16,900 have been found. The average result of 56 examinations of Boston sewage from July to December, 1903, showed 5,430,000 bacteria per c.c., at 20° and 3,760,000 per c.c. at 37°, of which 1,670,000 were acid formers. The average of 25 samples examined in July and August, 1904, showed 1,690,000 bacteria per c.c. at 20° and 1,400,000 at 37°; 429,000 per c.c. were acid formers (Winslow, 1905).

In unpolluted waters not only the absolute number of organisms developing at the body temperature is less, but its ratio to the gelatin count is very different. Rideal (Rideal, 1902) states that the proportion between the two counts in the case of a London water in a year's examination was on the average one to twelve. Mathews (Mathews, 1893) in 1893 gave the following figures, the contrast between the ponds and streams, which were presumably exposed to pollution, on the one hand, and the wells, springs, and taps, on the other, being marked.

Source of Water.	Average Number of Colonies per c.c.	
	Gelatin, 20°.	Wurtz Agar, 37.5°.
Wells, springs	1664	28
Reservoirs	153	43
Ponds	296	95
Taps	242	24
Streams	273	101

According to the English Committee appointed to consider the Standardization of Methods for the Bacterioscopic Examination of Water (1904), the ratio of the 20° count to the 37° count in good waters is generally considerably higher than 10 to 1. "With a polluted water this ratio is approached, and frequently becomes 10 to 2, 10 to 3 or even less."

In 1903 Nibecker and one of ourselves (Winslow and Nibecker, 1903) made an examination of 259 samples of water from presumably unpolluted sources in Eastern Massachusetts, including public supplies, brooks, springs, ponds, driven wells, and pools in the fields and woods, with a view to testing the value of the body-temperature examination. In many cases the samples showed high gelatin counts, since some of the waters were exposed to surface wash from vacant land, but the average number of organisms developing on lactose agar at 37 degrees was less than 8 per c.c., as will be seen by reference to the table on the following page. The highest individual counts obtained were 95 in a meadow pool, 83 in a brook, and 74 in a barnyard well, the latter probably actually polluted. Only two samples in the whole series, one from the well above mentioned, gave any red colonies on the agar plates.

For a series of shallow surface wells recently examined by one of us (S. C. P.) a similar relation is indicated in the table on page 69; 124 samples which showed no colon bacilli and were apparently unpolluted, gave an average of 190 bacteria per c.c. at 20° and 8 at 37° with less than one red colony per c.c.; 23 samples which did contain colon bacilli averaged 570 bacteria

RELATION OF 20° AND 37° COUNTS IN SAMPLES OF WATER FROM APPARENTLY UNPOLLUTED SOURCES

(WINSLOW AND NIBECKER, 1903)

Source of Samples.	Number of Samples.	Gelatin Plates, 20°.	Litmus-lactose-agar Plates, 37°.		Dextrose-Broth Tubes.			
		Average Number of Colonies.	Average Number of Colonies.	Plates Showing Red Colonies.	Number of Tubes.	Number of Tubes with Gas.	Number of Tubes with Gas 1–0.	Number of Tubes with Gas 2–1.
Cambridge supply (tap)	5	94	11	0	15	0	0	0
Wakefield and Stoneham supply (tap)	7	59	6	0	21	0	0	0
Lynn supply (tap)	6	16	3	0	18	0	0	0
Brookline supply (tap)	1	..	18	2	3	0	0	0
Plymouth supply (tap)	6	35	2	0	18	0	0	0
Peabody supply (tap)	3	141	21	0	9	2	2	0
Dedham supply (tap)	6	3717	1	0	18	0	0	0
Newburyport supply (tap)	6	36	9	0	18	0	0	0
Salem supply (tap)	5	232	14	0	15	0	0	0
Taunton supply (tap)	4	13	2	0	12	0	0	0
Sharon (well) (tap)	3	738	46	2	9	3	0	3
Medford supply (tap)	5	524	8	0	15	0	0	0
Milton supply (tap)	2	4700	0	0	6	0	0	0
Westerly, R. I., supply (tap)	1	..	12	0	3	0	0	0
Brooks	61	223	7	0	183	13	13	0
Driven wells	15	18	1	0	45	0	0	0
Springs	32	294	2	0	95	13	13	0
Ponds fed by brooks	15	167	9	0	45	1	1	0
Melted snow	1	..	4	0	3	0	0	0
Pools in fields	22	365	31	0	66	2	2	0
Pools in woods	22	181	3	0	65	0	0	0
Roadside pools	10	811	4	0	30	2	2	0
Stream, Blue Hill Reservation	1	..	0	0	3	0	0	0
Flow from rocks	2	47	0	0	6	0	0	0
Ponds fed by springs	6	188	2	0	18	0	0	0
Drainage from manured pasture	1	1235	27	0	3	0	0	0
Swamps	3	269	6	0	9	5	5	0
Rain-water after twelve hours' heavy fall	7	..	2	0	21	0	0	0
Shallow well in Lynn woods	1	15	1	0	3	0	0	0
Totals	259	..	..	4	775	41	38	3

per c.c. at 20° and 55 at 37° with an average of 7 red colonies.

BACTERIAL CONTENT OF 147 SHALLOW WELLS

PERCENTAGE OF SAMPLES IN EACH GROUP

		Bacteria per c.c.								
B. coli.		0	1–10	11–20	21–50	51–100	101–500	501–1000	1001–2000	2001–3000
−	Gelatin, 20°	3	16	14	16	11	31	5	4	
+		..	..	5	...	10	57	10	14	5
−	Agar, 37°	15	63	10	10	1	1			
+		..	31	35	22	4	4	4		
−	Red colonies	86	12	2						
+		30	52	9	9					

Significance of High Temperature Counts. Important data as to the distribution of bacteria which will develop at high temperatures may be found in a paper by Gage (1906), coupled with a suggestive discussion of the general significance of bacterial ratios. The table on page 70 shows some of the most significant results obtained by plating waters of various degrees of purity at 20°, 40° and 50°. We have rearranged the lines of the table so as to make the progression from more to less polluted waters a fairly regular one. The colony count at 50° shows an even sharper differentiation than that at 40°. Gage rightly concludes that "the information to be obtained by counts of bacteria and acid-producing organisms at any one of the above temperatures is greatly increased by the combination of the results obtained from counts at two or more temperatures."

AVERAGE NUMBER OF BACTERIA AND ACID-PRODUCERS DEVELOPING AT 20°, 40°, AND 50° C., WITH DIFFERENT CLASSES OF WATERS

GAGE (1906) REARRANGED

	Bacteria per c.c.			Acid-producing Bacteria.		
	20° C. 4 D.	40° C. 24 Hrs.	50° C. 24 Hrs.	20° C. 4 D.	40° C. 24 Hrs.	50° C. 24 Hrs.
Sewage	2,990,000	557,500	7,700	1,940,000	346,000	4,400
"	1,676,000	360,000	29,500	1,032,000	283,000	24,900
Septic effluent	485,000	126,500	410	241,000	90,000	240
Contact effluent	146,600	26,100	8,300	112,400	22,700	8,000
" "	389,000	59,300	8,000	292,000	45,000	8,000
" "	306,000	89,600	485	193,000	46,000	200
Trickling filter effluent	15,500	1,730	154	15,200	1,360	100
Do	23,300	2,030	54	16,000	1,180	20
Canal water	16,400	112	5	6,700	87	2
River water	16,900	207	4	2,500	134	2
Settled canal water	2,800	212	2	1,650	66	1
Sand filter effluent (sewage)	1,640	1,375	2	2,360	1,195	1
Do	35	4	0	29	2	0
Do	1,300	130	1	345	119	0
Do	670	170	2	1,045	154	0
Water filter effluent	32	3	1	6	1	1
Do	715	170	2	259	101	1
Do	62	1	0	16	0	0
Do	150	22	1	14	17	1
Do	64	5	1	11	3	1
Shallow well	1,000	2	0	3	1	0
" "	507	72	0	82	55	0
Pond	27	1	0	8	1	0
"	71	8	0	30	5	0
Spring	49	0	0	6	0	0
"	80	2	0	8	2	0
Driven well	41	0	0	0	0	0

In warm weather the interpretation of the body-temperature count must be made less rigid than at other seasons. Recent investigations have shown that

in midsummer bacteria capable of growth at 37° are more abundant in normal waters than in winter and spring.

Winslow and Phelps examined 86 samples from springs, wells, brooks and pools during the winter and spring months and found only 12 which showed more than 25 bacteria per c.c. and only 3 which showed more than 100 per c.c. on lactose-agar. On the other hand, of 58 samples from corresponding sources examined in summer, 16 contained more than 100 bacteria per c.c. A series of 20 pools, ponds, and brooks at Mt. Desert, Me., which were entirely free from human or animal pollution, were examined in the late summer of 1906. Only 4 of the 20 samples gave

20° AND 37° COUNTS OF RAW WATER AT WILMINGTON FILTER PLANT

(WHIPPLE, 1913)

Month.	1908.			1909.		
	Bacteria per c.c.		Per Cent	Bacteria per c.c.		Per Cent.
	Gelatin, 20°.	Bile-agar, 37°.		Gelatin, 20°.	Bile-agar, 37°.	
January	4630	124	2.7	3880	94	2.3
February	6830	358	5.3	4800	260	5.4
March	8800	350	4.0	4620	387	8.4
April	3170	149	4.7	5080	347	6.7
May	2010	119	5.9	3340	229	6.8
June	1640	241	14.7	2350	158	6.7
July	3150	432	13.7	2940	57	1.9
August	3140	451	14.4	1430	230	16.1
September	3400	644	18.9	2620	619	22.8
October	5180	439	8.5	1380	129	9.4
November	6850	78	1.2	1650	97	3.9
December	4100	203	4.9	4150	194	4.7

counts under 25 at 37°, and 7 of them gave counts over 100, the highest figure being 425.

Whipple (1913) gives some figures for the raw water at the Wilmington, Del., filter plant (page 71) which bring out the seasonal variation very clearly.

Another special case in which the ratio between the 20° and the 37° count fails to be significant is that of a water which has been treated with bleaching powder. Most of the bacteria which survive chlorine treatment are of course spore formers, many of them belonging to the hay bacillus group, and it happens that most of these spore formers can grow at body temperature. Thus it is common to get counts as high at 37° as at 20° with such waters, although the absolute numbers are generally small. This point is illustrated in the two tables below, showing the results of experimental treatment of Merrimac River water at the Lawrence Experiment Station and of swimming pool water at the University of Wisconsin.

COMPARATIVE EFFECTS OF CHLORINE DISINFECTION UPON 20° AND 37° COUNTS, MERRIMAC RIVER WATER, AT LAWRENCE, MASS.

(Clark and Gage, 1909)

Sample.	Bacteria per c.c.			
	Untreated Water.		Treated Water.	
	20°.	37°.	20°.	37°.
A	3,400	30	12	4
B	28,900	130	4	4
C	14,000	75	35	47
D	3,700	81	43	62

COMPARATIVE EFFECTS OF CHLORINE DISINFECTION UPON 20° AND 37° COUNTS, SWIMMING POOL WATER AT UNIVERSITY OF WISCONSIN

(TULLY, 1912)

Sample.	Bacteria per c.c.			
	Untreated Water.		Treated Water.	
	20°.	37°	20°.	37°.
A	275	16	0	1
B	445	480	4	5
C	920	483	8	8
D	5,630	680	4	2
E	19,100	1,140	30	45
F	24,000	1,190	130	120
G	10,000	1,080	14	27
H	1,700	690	15	9
I	2,570	780	12	30
J	2,800	560	27	66

Under ordinary conditions it is clear that organisms growing at the body temperature and those fermenting lactose are not numerous in normal waters. The absolute count at 37° seldom exceeds 50, and is rarely over 10 per cent of the 20° count, except after hot periods in the late summer; acid producers are generally entirely absent. On the other hand, the numbers on the litmus-lactose-agar plate will be likely to run into hundreds with a good proportion of red colonies when polluted waters are examined.

CHAPTER V

THE ISOLATION OF SPECIFIC PATHOGENES FROM WATER

The discovery of the organisms which specifically cause infectious diseases naturally led to the hope that their isolation from polluted water might become the most convincing proof of its sanitary quality. The typhoid bacillus and the spirillum of Asiatic cholera were in this connection of paramount importance, and to the search for them many investigators have devoted themselves.

The Search for Typhoid Bacilli. In the earlier examinations of water for the typhoid bacillus an attempt was made to use media which especially favored the growth of the microbe sought for, or to begin with some process of "enrichment" in which the sample was incubated under conditions which would favor the growth of the pathogenic organisms while checking the development of the common water bacteria. It was apparent that the body temperature and the presence of a slight excess of free acid furnished such conditions, and most of the methods suggested rest upon these principles. Among them, one of the earliest was that of Parietti (Parietti, 1890), which consists in the addition of the water to a series of broth tubes,

containing increasing amounts of a solution of 4 per cent hydrochloric acid and 5 per cent phenol. From tubes in which growth occurs after 24 hours at 37 degrees the organisms present may be isolated in pure cultures by some plating method and identified by subcultures.

The great difficulty with a majority of the enrichment processes is that the conditions which favor the multiplication of the typhoid bacillus are frequently suited in an even higher degree to B. coli and other intestinal organisms. Being present in almost all cases in much higher numbers than B. typhi, these bacteria develop more abundantly, and effectually mask any disease germs originally present. In order to obviate this difficulty, Hankin (Hankin, 1899), after adding successively increasing portions of Parietti solution to tubes inoculated with the water to be tested, selected the second highest tube of the series in which growth occurred for the inoculation of a new set, finally plating as above. He believed that the chance for overgrowth by this method is somewhat decreased; but in the hands of other investigators it has not met with marked success. Klein (Thomson, 1894) in his investigations, made use of the Berkefeld filter to concentrate the organisms in the sample. Some observers abandoned the enrichment process altogether and recommended direct plating upon solid media such as phenolated gelatin or the Elsner (Elsner, 1896) medium, made by adding 10 per cent of gelatin and 1 per cent of potassium iodide to an infusion of potato whose reaction has been adjusted to 30 on Fuller's scale.

In the last five years considerable progress has been

made in the development of new methods for isolating the typhoid bacillus. These fall in three distinct groups: first, the direct isolation by differential, frequently colored, solid media; second, isolation as above, preceded by enrichment methods; third, isolation, with or without enrichment, preceded by concentration of the organisms by agglutination with typhoid serum or concentration by chemical precipitation.

Isolation Methods, Using Solid Media. Drigalski and Conradi (Drigalski and Conradi, 1902), prepared a medium primarily for the isolation of typhoid bacilli from excreta, which may also be applied in water bacteriology. This consists of an agar medium containing nutrose, sodium chloride, litmus, lactose, and a dye, " crystal violet "; and it is used in the form of plate cultures infected by smearing the surface with a bent glass rod after thorough cooling. The culture medium is a selective one, ordinary saprophytes failing to grow, while after 14 to 24 hours at 37°, colon and typhoid colonies can be readily distinguished from one another. The colon bacillus produces red, non-transparent colonies, of variable size and depth of color, while the typhoid colonies are blue or violet, transparent and of smaller size, seldom exceeding three millimeters in diameter. (For formula, see p. 275.)

Endo (Endo, 1904) has suggested the use of a fuchsin-lactose-agar decolorized by sodium sulphite. Upon this medium B. coli produces bright red, sharply defined round colonies in 24 hours at 37°, while B. typhi gives round, colorless, transparent colonies with thin margins. This medium has been somewhat modified

by Gaehtgens (Gaehtgens, 1905) by the addition of caffein, and he found it of great service in isolating the typhoid bacillus from stools of patients suffering with the disease. No attempts were made by him to isolate the organism from polluted water. (For formula, see page 276.)

Loeffler (Loeffler, 1903 and 1906) and Lentz and Tietz (Lentz and Tietz, 1903 and 1905) have made use of an agar medium containing malachite green. This medium is supposed to inhibit the growth of B. coli while favoring B. typhi, and has been recommended for the isolation of the organism from fæces. Dœbert (Dœbert, 1900) has shown that certain varieties of malachite green are not suited to this purpose. Nowack (Nowack, 1905) has also pointed out the same fact, and ascribed the difference to the presence of dextrin. He also showed that a medium 0.8 per cent alkaline to phenol-phthalein is more favorable to B. typhi and less favorable to B. coli than one neutral to litmus. With such a medium he found that about 20 per cent of the typhoid bacilli present develop. Lemke (1911) has recently reported good success in isolating typhoid and para-typhoid bacilli from artificially infected waters by the use of nutrient broth containing 3–5 per cent of sodium chloride and varying amounts of malachite green as an enrichment medium.

Torrey (Journal of Infectious Diseases, 1913, XIII, 263) and Krumwiede and Pratt (Journal of Experimental Medicine, 1914, XIX, 501) find that media made up with brilliant green are of great value in isolating typhoid and paratyphoid bacilli from stools.

Dr. Chas. Krumwiede of the Research Laboratories of the N. Y. City Department of Health has very courteously furnished us with the following description of the use of the brilliant green agar, which should prove of value in practical water work.

The medium is made up as follows:

Composition of medium:

Agar	1.5%
Liebig's extract of beef	0.3%
Witte's peptone	1.0%
Sodium chloride	0.5%
Lactose	1.0%
Glucose	0.1%
Andrade indicator	3.0%
Brilliant green (in 0.1% sol.)	0.2 cc. to each 100 c.c. (1–500,000).

Andrade indicator:

100 c.c. .5% watery solution of acid fuchsin,
16 c.c. normal NaOH solution.

The solution, after the addition of the alkali should turn slowly to a salmon yellow; if it does not, a little more alkali may be added.

Dr. Krumwiede describes the use of the medium as follows:

"The reaction should be set to the indicator, so that the agar when hot is pinkish to red, but not deep

red. On cooling, the color should disappear. The change of color adjusts itself slowly; that is, if acid is added, the pink or red color desired may develop immediately but will fade after a few minutes, more acid being then required.

"The brilliant green should not be added until the reaction is adjusted, or, if the agar is kept in stock, it should be added just before pouring the plates. We have also been adding the sugars, in 25 per cent solutions, just before pouring.

"The most convenient method is to have the agar in bottles of 100 c.c. each, from each of which we make six plates only, as the layer of agar should be thick.

"After pouring, the plates should be left open till the agar is hardened, and then covered with porous covers to absorb the water of condensation. Unless the plates are absolutely dry, diffusion becomes troublesome.

"We have added 0.1 per cent glucose, which has the effect of markedly increasing the size of the typhoid colonies, but has the disadvantage of causing some color to appear in them, especially if they are closely packed. As the typhoid colonies are rather characteristic (when glucose is present), this does not as a rule interfere. Furthermore, it gives plenty of material for direct agglutination of the colonies (macroscopic slide method).

"If one green dye plate is inoculated with the amount that will give discrete colonies on two successive Endo

plates, and another green plate with twice this amount the distribution of colonies is usually satisfactory. (By 'two successive Endo plates' is meant streaking two plates without burning off the platinum spatulum.) Sometimes even this amount will give sterile or nearly sterile plates, but usually one of the plates will be neither overcrowded nor sterile.

"The use of 1.5 per cent agar allows some diffusion of the color, but as most of the lactose-fermenting types are inhibited, this is not as a rule troublesome. Three per cent agar has been tried, but the dye, in the concentration used for the 1.5 per cent agar. does not act under these conditions. Any change in the constituents of the medium will necessitate a readjustment of the concentration of the dye.

"The sample dye employed is Brilliant Green Extra Crystals No. 366, from Bayer, 117 Hudson Street, New York City. The concentrations of the dye may have to be varied with different samples.

"Sixty-six stools known to contain typhoid bacilli (normal stools to which had been added small amounts of carrier stools), were examined in parallel on Endo and brilliant green agar with the following results:

Endo.....	30 positive	36 negative	45% positive
Green Agar...	57 positive	9 negative	86% positive."

The use of the inhibitive anilin dyes like crystal violet and malachite green has the disadvantage of also inhibiting to some extent the development of the weaker

typhoid organisms. Another principle is involved in the media proposed by Hiss and Hesse. These are both agar media of lower spissitude than ordinary agar and the separation of the typhoid and colon groups of organisms depends on the greater motility of the former and their tendency to swim out from the colonies and form branch-like processes or turbid zones on a semi-solid medium. The Hiss medium is made up as follows (Hiss, 1902):

Agar	15 gm.
Gelatin	15 gm.
Liebig's meat extract	5 gm.
NaCl	5 gm.
Dextrose	10 gm.
Distilled water	1000 cc.

Reaction 1 per cent normal.

The Hesse medium has been used with great success by Jackson and Melia (1909). Its general composition is as follows:

Agar	5 gm.
Witte's pepton	10 gm.
Liebig's meat extract	5 gm.
NaCl	8.5 gm.
Distilled water	1000 c.c.

Reaction 1 per cent normal.

Jackson (1909) recommends that the agar used should be dried for half an hour at 105°, and under

these circumstances 4.5 grams may be used in the formula instead of the 5 grams recommended by Hesse. The medium must be stored in an ice chest with saturated atmosphere. Plates must be made in sufficient dilution to give a few colonies on the plate; and where this is done the typhoid colonies are sharply distinguished from those of B. coli by the fact that they grow to a considerable size, often several centimeters in diameter and show a broad translucent or scarcely turbid zone between the white opaque centre or nucleus and the perfectly circular narrow white seam or edge.

Stokes and Hachtel (1912) have suggested a modification of the Hesse medium which consists in the increase of the agar to 5.5 per cent and in the addition of 10 gm. of lactose and 50 gm. of glycerin to the formula cited above. The agar is dried out at 105 degrees for half an hour and dissolved in half a liter of water. The meat extract is added to the other half liter and freed from muscle sugar by inoculation with the colon bacillus and incubation for 24 hours at 37 degrees. The sugar-free broth thus prepared is filtered and to it is added the peptone, lactose and salt. The two half liter portions of the medium are then mixed and boiled for 30 minutes. The medium is filtered and adjusted to a neutral reaction, the glycerin is added and the medium is tinted with azolitmin solution before tubing and sterilizing. Typhoid and para-typhoid organisms develop medium-sized, pinkish colonies with concentric rings, and may thus be distinguished from colonies of B. alcaligenes, B. proteus and other motile forms. Organ-

isms of the B. subtilis group must be eliminated by microscopic examination, using the Gram stain.

Preliminary Enrichment. In most cases plate isolation is preceded by some sort of enrichment process designed to favor the typhoid bacilli at the expense of the members of the B. coli group. The original use of carbol broth has been already discussed. In Europe caffein media have been used for this purpose and in the United States bile media have been strongly recommended.

The important fact that caffein has an inhibitory action on colon bacilli, announced by Roth (Roth, 1903) has given rise to much investigation, and offers one of the most promising methods for the isolation of the typhoid bacillus from water. Hoffman and Ficker (Hoffman and Ficker, 1904) developed methods for the isolation of B. typhi from fæces and from infected water by its use in connection with nutrose and crystal violet. For the isolation from infected water solutions were prepared as follows:

1. Ten grams of nutrose dissolved in 80 c.c. of sterilized distilled water.

2. Five grams caffein, in 20 c.c. sterilized distilled water.

3. One-tenth gram of crystal violet in 100 c.c. water. Solutions 1 and 2 were mixed by shaking together in a flask, and the mixture poured into a flask containing 900 cubic centimeters of the water to be tested; 10 c.c. of solution 3 were gradually added, and the whole thoroughly mixed by shaking and then incubated at

37 degrees for not over 12–13 hours. At the end of the incubation period loopfuls of the solution were smeared over Drigalski-Conradi plates.

By this method the B. typhi was isolated from mixtures in river water containing one typhoid bacillus to 51,867 water bacteria and colon bacilli.

A number of investigations have shown that the action of the caffein is not as markedly selective as at first claimed. Kloumann (Kloumann, 1904) obtained no better results by this method than by the Drigalski-Conradi medium alone, and Willson (Willson, 1905) found that certain strains of B. typhi were inhibited, while strains of B. coli developed feebly in the presence of 0.5 per cent of caffein.

In this country marked success in the isolation of the typhoid bacillus has been attained by the use of lactose bile as an enrichment medium. Jackson (Jackson and Melia, 1909), the principal exponent of this procedure, recommends that sterilized undiluted fresh ox gall (or an 11 per cent solution of dry fresh ox gall) containing 1 per cent of peptone and 1 per cent of lactose be made up in 40 c.c. amounts in fermentation tubes to which varying amounts of water, up to 10 c.c. may be added. After incubation for 48 or 72 hours, he plates on Hesse agar and he finds that in the bile medium B. typhi tends to overgrow B. coli while most other organisms are entirely suppressed.

Dr. Krumwiede has found brilliant green broth useful as an enrichment medium for the isolation of

typhoid bacilli from stools. In a personal letter he says: "If stools are inoculated into brilliant green broth (extract broth neutral to phenolphthalein with 1 per cent of glucose), there is a suppression or inhibition of many of the lactose fermenters. The dye, even in strong solutions, has little effect on the growth of pyocyaneus, proteus, paratyphoid-like (intermediates), and aërogenes (mucosus types, non dulcit fermenters) bacilli. Typhoid bacilli will multiply in dilutions of the dye that inhibit the colon types. As the fæces itself reduces the activity of the dye, adjustment is necessary and can be obtained by inoculation of 0.1 c.c. of the fæcal suspension into broth containing 1–300,000, 1–400,000 and 1–500,000 of the dye. These tubes, after 15 to 18 hours' incubation, are plated on Endo."

Concentration by Agglutination or Precipitation. A physical concentration of the typhoid bacilli precedes enrichment or isolation in the procedure recommended by many authors. Klein, as noted above, accomplished this by passing the water through a Berkefeld filter. Other workers have made use of agglutination or chemical precipitation for the same purpose.

The phenomenon of agglutination was made the basis of a method of isolating B. typhi from water by Adami and Chopin (Adami and Chopin, 1904). Two-liter samples of the water were collected in sterilized bottles (Winchester quarts), and to each was added 20 c.c. of 1 per cent glucose broth. The sample was incubated for 18 to 24 hours at 37° C., after which

10 c.c. portions were withdrawn and placed in long, narrow test tubes. To each of these tubes enough typhoid serum of known potency was added to make a regularly graded series, 1–50, 1–100, 1–150, and 1–200. The probable presence of the typhoid bacillus was manifest by the formation of flocculi within a quarter of an hour, and agglutination was complete in from 2 to 5 hours.

The tube having the greatest dilution in which agglutination was apparent was then examined by breaking off the lower end, containing the precipitate, washing the sediment two or three times with sterile water after removing the clear supernatant liquid, and allowing the bacteria to settle again. The organisms remaining were plated upon various media, and examined biochemically to determine the true character of the suspected colonies. It was found that a dilution of 1 to 60 was the highest which could be used with the organisms examined, and it is therefore probable that high dilutions (greater than 1–60) cannot be successfully used.

Investigation of an organism isolated by this method was made by Klotz (1904), who found the culture to be not a typical B. typhi, but a form showing certain points of similarity to both B. typhi and to B. coli, and probably intermediate between them. Frost (1910) isolated a bacillus of the B. proteus group from filtered Potomac water which agglutinated with typhoid serum in high dilutions. As Klotz points out, therefore, it is evident that even when a positive result is obtained with a relatively high dilution of

typhoid serum, the action may by no means be absolutely specific.

Schepilewski (Schepilewski, 1903) and Altschuler (Altschuler, 1903) have also used agglutination as a means of precipitating the bacteria after enrichment cultivation in broth. The former incubated the culture at 37° for 24 hours, then added a serum of high potency, allowed the mixture to stand for 2 to 3 hours, and then centrifuged. The supernatant liquid was removed, and the mass of agglutinated cells broken up by shaking with glass beads and salt solution. Upon plating upon litmus lactose agar the organisms could be detected. In this way positive isolation was made from water containing 1 loopful of a broth culture in 50 liters of water. Altschuler's method of enrichment was essentially like that of Schepilewski. From the surface of the culture developed at 37°, 10 c.c. were removed to a tapering tube provided with a rubber tube at the bottom. Serum was added in the proportion of one part in 50, the culture agitated to release entangled non-agglutinated bacilli and the sediment run into a tube containing 1 per cent peptone and 0.5 per cent salt. The agglutinated mass was broken up by shaking with sand, and the culture incubated at 37° for 24 hours, then plated on Drigalski-Conradi plates. The organism was isolated from dilute suspensions in water (150 in 1 liter) and also from the fæces of a typhoid patient from which other methods gave negative results.

Precipitation Methods. A number of methods for concentrating typhoid bacilli in water by chemical

precipitation have been tested experimentally, with some degree of promise. Vallet (Vallet, 1901) was the first to employ this principle, and made use of sodium hyposulphite and lead acetate. The mixture was then centrifuged and the precipitate dissolved in more hyposulphite. The clear solution was then plated.

Schüder (Schüder, 1903) observed that the lead salt reacted harmfully upon the bacteria, and that the hyposulphite should be in excess. In his experiments water was allowed to stand in tall jars for 24 hours. To 2 liters of infected water, 20 c.c. of a 7.75 per cent solution of sodium hyposulphite was added, and after thorough mixing 20 c.c. of a 10 per cent solution of lead nitrate. The precipitate, after 20 to 24 hours, was treated with 14 c.c. of saturated sodium hyposulphite solution and shaken. From the clear solution 0.2 to 0.5 c.c. portions were streaked upon Drigalski-Conradi plates which were then incubated at 37° for 24 hours. Ficker (Ficker, 1904) modified the process still more by using ferric sulphate, and dissolved the precipitate with neutral potassium tartrate. The final solution was then plated on Drigalski-Conradi medium. Ficker claimed that this method gives excellent results, 97–98 per cent of the typhoid bacteria being carried down with the precipitate.

Müller (Müller, 1905), in comparing different precipitation methods, adopted ferric oxychloride as the most suitable precipitant, because of its quicker and less destructive action. Willson (Willson, 1905) suggested the use of alum as a precipitant. He added 0.5 gr. alum per liter of water examined. The mixture

was then centrifuged, and the precipitate suspended in a small amount of water and plated on Drigalski-Conradi medium. Nieter (Nieter, 1906) made 20 parallel experiments, using very pure water infected with typhoid bacilli in varying numbers. By precipitating with ferric sulphate and sodium hydrate, centrifuging, and then filtering through a sterile filter he obtained small numbers of bacteria. Using iron oxychloride as the precipitant, he confirmed the results of Müller. By plating on malachite green agar he was often able to get positive results when the Drigalski-Conradi medium failed.

By use of a combination of enrichment and chemical precipitation, Ditthorn and Gildemeister (Ditthorn and Gildemeister, 1906) isolated the typhoid bacillus from enormous artificial dilutions in water. In the typhoid fever epidemic in Posen, in 1906, it was found that the bile of those dying from the disease contained nearly pure cultures of typhoid bacilli. This led the authors mentioned to use bile and bile agar as enrichment media. After precipitating by Müller's method, the whole of the precipitate was added to 100 c.c. sterile ox bile and grown at 37° for 24 hours, after which time 1 c.c. portions were plated. With extreme dilutions it was found desirable to incubate for 48 to 72 hours. The results were unsatisfactory in the presence of large numbers of water bacteria. It is also pointed out that the iron oxychloride is bactericidal in 48 hours.

Separation on the Basis of Motility. Drigalski (Drigalski, 1906) has suggested the separation of B. typhi

from other bacteria in water through its greater motility. He succeeded in isolating typhoid bacilli from two springs by the following method: 5 to 10 liters of water were allowed to stand one to two days in tall milk cans at room temperature. Samples were taken from the surface and plated on litmus-lactose agar (Drigalski-Conradi medium), the amount of water to be used varying with the contamination.

Starkey (1906) has suggested the use of an apparatus consisting of a piece of glass tubing bent so as to give four successive connected loops. This is filled with phenol broth, inoculated at one end, and incubated anærobically. The more actively motile bacilli find their way to the fourth loop from which they may be isolated by plating.

Review of Suggested Procedures. The methods of examining water for B. typhi may be conveniently summarized as follows:

- Examination of water for typhoid bacilli
  - 1. Physical concentration
    - *a.* By filtration
    - *b.* By agglutination
    - *c.* By chemical precipitation
      - Schüder's process
      - Fischer's process
      - Willson's process
      - Müller's process
  - 2. Enrichment
    - *a.* Parietti's carbol broth
    - *b.* Jackson's lactose bile
    - *c.* Hoffmann and Ficker's caffein process
    - *d.* Brilliant green broth
  - 3. Isolation
    - *a.* Elsner's gelatin medium
    - *b.* Endo's medium
    - *c.* Loeffler's malachite green medium
    - *d.* Brilliant green agar
    - *e.* Drigalski-Conradi agar
    - *f.* Hiss's medium
    - *g.* Hesse's medium
  - 4. Identification
    - *a.* Morphological and cultural characters
    - *b.* Agglutination

Of the comparative advantages of these methods it is still too early to speak with finality. Up to the present time the use of caffein and lactose bile has apparently been followed by the best results, and it seems likely that of the precipitation methods that employing the oxychloride of iron is the best. The successful use of malachite green and brilliant green in the isolation of typhoid and paratyphoid bacilli from fæces suggests that the more general adoption of these media in water examinations would possibly prove fruitful.

Identification of the Typhoid Bacillus. At the end of the process the identification of the pure cultures isolated is again subject to considerable uncertainty. The typhoid bacillus belongs to a large group which contains numerous varieties differing from each other by minute degrees. The inability to reproduce the disease by inoculation in available test animals owing to their natural immunity is a serious drawback; and the specific biochemical characters of the organism are, as it happens, mostly negative ones, as shown by comparison with B. coli, to which it is supposed to be allied.

COMPARISON OF THE CHARACTERS OF B. COLI AND B. TYPHI

(Horrocks, 1901)

B. COLI	B. TYPHI
(1) Surface Colonies, Gelatin Plates.—Thicker, and grow more rapidly than those of B. typhi. After forty-eight hours' incubation at 22° C. they are usually large and characteristic.	(1) Much thinner than those of B. coli, and grow more slowly. After forty-eight hours' incubation at 22° C. they are hardly visible to the naked eye.

(2) Gelatin-stab.—Quick growth on the surface and along the line of inoculation.	(2) Slow growth on the surface like the colonies; along the line of inoculation, the growth is much thinner, and often ends below in a few white points consisting of discrete colonies.
(3) Gelatin-slope.—Thick, broad grayish-white growth with a crenated margin.	(3) Thin narrow grayish-white growth, crenated margin not marked as in B. coli.
(4) Witte's Peptone and Salt Solution.—Indol produced.	(4) No formation of indol.
(5) Milk.—Coagulated.	(5) Unchanged after a month.
(6) Litmus-whey, one week at 37° C. Acid produced usually requiring from 20 to 40 per cent of N/10 alkali to neutralize it.	(6) Very small amount of acid produced, requiring not more than 6 per cent of N/10 alkali to neutralize it.
(7) Neutral-red Glucose-agar.—Marked green fluorescence.	(7) No change.
(8) Glucose-gelatin and Lactose-gelatin Shake Cultures, and Glucose-agar-stab.—Marked gas formation.	(8) No gas formation.
(9) Gelatin, 25 per cent, incubated at 37° C. — Thick film appears on the surface.	(9) No film appears on the surface, but a general growth takes place throughout the tube.
(10) Potato.—As a rule, a thick yellowish-brown growth.	(10) Thin transparent growth hardly visible to the naked eye.
(11) Proskauer and Capaldi's Media. No. I, after twenty hours' growth, medium acid. No. II, Growth, medium neutral or faintly alkaline.	(11) No. I, no growth or change in the reaction of the medium. No. II, Growth, medium acid.
(12) Nitrate-broth.—Nitrate reduced to nitrite.	(12) Reduction of nitrate not so marked.
(13) Microscopical Appearances.—A small bacillus often like a coccus, not motile as a rule.	(13) Usually longer than B. coli; highly motile, with a quick serpent-like movement.
(14) Flagella.—Usually 1 to 3, short and brittle; sometimes 8 to 12, long and wavy.	(14) Usually 8 to 12, long and wavy.
(15) Agglutination.—As a rule, no agglutination with a dilute anti-typhoid serum.	(15) Marked agglutination with dilute anti-typhoid serum.

In addition to the biochemical characters noted above the typhoid bacillus is characterized by its failure to produce gas and by its feeble acid production in lactose

media and by its characteristic colonies on Drigalski-Conradi agar, Endo medium, Hiss medium, and Hesse agar. In studying its immunity reactions agglutination should in all important cases be supplemented by the Pfeiffer reaction and the absorption test which will be found described in standard text-books on bacteriology.

Of the many observers who have reported the isolation of the typhoid bacillus from water, all but the most recent are quite discredited, on account of the insufficiency of the confirmatory tests, and even the latest results should be received with caution. Since the introduction of the Widal (Widal, 1896) reaction, founded on the fact that typhoid bacilli examined under the microscope in the diluted blood-serum of a typhoid patient lose their motility and " agglutinate " or clump together, an important aid has been furnished in the diagnosis. Yet serum tests are notably erratic, and insufficient to identify an organism without an exhaustive study of biochemical reactions. Many organisms are agglutinated by typhoid serum in a more or less dilute solution, and agglutinations are not significant unless obtained in dilutions as great as 1–500 or 1–1000. The discovery of the Bacillus dysenteriæ of Shiga,[1] which closely resembles the typhoid bacillus, has made the identification of the latter more dubious than ever. Hiss (1904) has shown that the fermentation and agglutination reactions of the two organisms are in many respects alike, and Park and his

* For an account of biology of B. dysenteriæ the student is referred to an article by Dombrowsky, 1903.

associates (1904) have shown that there are not less than three distinct types of dysentery bacilli forming that group.

Isolation of Typhoid Bacilli from Water. The methods we have been discussing have many of them been used chiefly for obtaining the typhoid bacillus from fæces, which is much easier than its isolation from polluted water. There are, however, a number of cases in which the organism has undoubtedly been isolated from polluted water, as by Kübler and Neufeld (Kübler and Neufeld, 1899), who examined a farmhouse well at Neumark in 1899, and Fischer and Flatau (Fischer and Flatau, 1901), who discovered an organism responding to a most exhaustive series of tests for the typhoid bacillus in a well at Rellingen in 1901. In these cases the water was directly plated upon Elsner's medium or phenolated gelatin with no preliminary process of enrichment. Willson (Willson, 1905) summarized the instances in which the typhoid bacillus had been isolated from infected drinking water, up to 1905, and included, in addition to the above-mentioned cases, the following:

1. By Lösener, in 1895, from the Berlin water supply.

2. By Conradi, in 1902, from a well at Pecs in Hungary, by use of carbol gelatin plates.

3. By Jaksch and Rau, in 1904, from the water supply of Prague, and also from the river Moldau, by caffein-nutrose crystal violet agar.

4. By Ströszner, in 1904, from a well near Budapest, by the same method.

Several other instances in which the isolated organ-

isms gave positive agglutination tests, as well as the usual cultural reactions, are also cited by Willson.

During the last 5 years a number of successful isolations of the typhoid bacillus have been reported in America. An organism obtained from the water-supply of Scranton, Pa., in 1907, by simple enrichment in Parietti bouillon, was identified as the typhoid bacillus by Prof. Fox after a very careful series of tests with immune sera (Pennsylvania, 1908). The most important results have been achieved, however, by Jackson with lactose bile enrichment and subsequent plating on Hesse agar. He reports the isolation of B. typhi from 10 c.c. samples of the Grass River at Canton, N. Y., and of a pond and stream at Hastings, N. Y., (both used as sources of water-supply) and from two 1 c.c. samples of the Hudson River near Hastings at the time of the typhoid epidemic there (Jackson and Melia, 1909). Stokes and Hachtel (1910) by the same method found organisms corresponding to typhoid in their general cultural reactions in four samples of surface-waters (two of them from an impounding reservoir of the Baltimore supply), in the sediment of a school well supposed to have caused typhoid fever, in a sewage-polluted stream and in two samples of market oysters. These organisms agglutinated with the blood of typhoid patients in 1/50 and 1/100 dilutions, but with an immune serum producing agglutination with a standard laboratory typhoid culture in dilution of 1/25,000 these water organisms would only agglutinate in dilutions of 1/250 or 1/500. Their identity must therefore be regarded as somewhat doubtful. The

same authors (Stokes and Hachtel, 1912) have more recently reported the isolation of the typhoid bacillus from the water in the neighborhood of a polluted oyster bed.

The search for the typhoid bacillus is usually suggested when an outbreak of the disease has cast strong suspicion upon some definite source of water-supply. By the time an epidemic manifests itself, however, the period of the original infection is long past, and the chances are good that any of the specific bacilli once present will have disappeared. While elaborate experiments have shown that B. typhi may persist in sterilized water for upwards of 2 months and in unsterilized water from 3 days to several weeks, the number of the organisms present is always very rapidly reduced. Even in highly polluted water their number is proportionately small; as is well shown by the experiments of Laws and Andrewes (Laws and Andrewes, 1894) who entirely failed to isolate the typhoid bacillus from the sewage of London and found only two colonies of the organism on a long series of plates made from the sewage of a hospital containing forty typhoid patients. So Wathelet (Wathelet, 1895) found that of 600 colonies isolated from typhoid stools and having the appearance characteristic of B. coli and B. typhi only 10 belonged to the latter species.

Epidemiological evidence confirms these results and indicates that the number of typhoid bacilli even in polluted water probably is never very great, while the fate of Lowell and Lawrence in 1890–91 and the more recent epidemics at Butler, Pa., and Ithaca, N. Y.,

demonstrate that even a small number of virulent organisms can bring about an almost wholesale infection. Indeed, if the virulent organism were as abundant as some results would indicate (Remlinger and Schneider, 1897), the human race would long since have been exterminated. A negative result in testing for typhoid bacilli has no significance and there is danger that it may be misinterpreted if the fact that it has been made comes to public knowledge. In spite of this danger, however, and in spite of the laborious and time-consuming nature of the process, the increasingly large number of positive isolations in recent years indicate that it is well worth trying in cases of special importance. The search for the typhoid bacillus should of course never supersede the examination for colon bacilli, since the latter are so much more numerous in water and so much more easily identified. Because of these facts, colon bacilli will continue to be our best index of pollution, while the positive isolations of the typhoid bacillus will supply additional proof of the deadly character of a water containing it.

Other Bacteria of the Typhoid Group Related to Intestinal Disease. The typhoid bacillus and the colon bacillus (which will be fully discussed in succeeding chapters) stand at the opposite ends of a series of many different varieties of organisms which are intermediate in their properties between B. typhi and B. coli, all being non-spore-forming, non-liquefying rods, which produce a more or less characteristic growth on solid media. Durham (1898) divided these forms into three main divisions, grouped, respectively, about B. typhi, B.

enteritidis and B. coli. Organisms of the first division ferment neither dextrose, lactose nor saccharose; those of the second ferment dextrose but not lactose; and those of the B. coli division form gas in both these sugars. The relationship of the commonest species is indicated in tabular form below:

BACTERIA OF THE COLON-TYPHOID GROUP

Species.	Dextrose.		Lactose.	
	Gas Formation.	Acid Production.	Gas Formation.	Acid Production.
B. alcaligenes	None	None	None	None
B. typhi	None	Slight	None	None
B. dysenteriæ	None	Distinct	None	None
B. enteritidis	Active	Strong	None	Slight
Paratyphoid bacilli	Active	Strong	None	Slight
Hog cholera bacillus	Active	Strong	None	Slight
B. coli	Active	Strong	Active	Strong

In the typhoid division, B. alcaligenes and B. dysenteriæ are the best-known forms, besides B. typhi itself. B. alcaligenes stands at the lower end of the whole series in fermentative power. B. typhi forms a slight initial acidity in milk and a slight acidity in dextrose broth, while the reaction of B. alcaligenes in sugar media is always alkaline. B. dysenteriæ, on the other hand, differs from B. typhi in the direction of the B. enteritidis group, producing a well-marked acid reaction, but no gas in dextrose media. B. typhi and B. dysenteriæ are, of course, also distinguished by their specific serum reactions.

The second great division of the colon-typhoid bacteria is the hog cholera group, or the Gärtner group, as Durham (1898) called it. As defined by him, it differed from the typhoid group by gas formation in dextrose, and from the colon group by the production of a final alkaline reaction in milk. It includes the Gärtner bacillus (B. enteritidis), the hog cholera bacillus (B. choleræ suis), and the paratyphoid bacilli. Some of these forms, the paratyphoid bacilli, for example, and B. enteritidis (isolated in cases of meat poisoning), produce intestinal disease in man.

There is no doubt that water is sometimes the means of distributing the germs of dysentery and diarrhœa, as shown by the decrease of these diseases in Burlington, Vt., (Sedgwick, 1902), and other communities where pure water-supplies have been substituted for polluted ones. Thresh (Thresh, 1903) described an epidemic of over 1000 cases of diarrhœa with 14 deaths, which occurred in England at Chelmsford and Widford, and was undoubtedly spread by the public water-supply. A somewhat similar epidemic of dysentery occurred in Warren and Kittanning, in Pennsylvania, in 1906, which was unquestionably due to contamination of the water, in this case a river-supply. It is possible that the examination of water for the B. dysenteriæ may in the future help to throw important light on the sanitary condition of a water.

Starkey (1909 and 1911) believes that all organisms giving the general reactions of the Gärtner and paratyphoid groups are significant and warrant the con-

demnation of a water-supply. The difficulty, however, is that while non-acid-forming bacteria of this general type are sometimes found in fæces, they are also found in other habitats, and they are less abundant proportionately, in polluted than in stored and safer waters. If true dysentery and paratyphoid bacilli can be isolated and identified by serum reactions it is, of course, highly important. Houston (1911), however, has recently tested the method suggested by Starkey (1906) for isolating these forms and found that it gave negative results even with a water artificially infected with about 14 typhoid bacilli and 21 Gärtner bacilli per c.c. In his own studies Houston reports that in the examination of 13,442 microbes from polluted river water he found only one member of the Gärtner group; and in another study of 20,771 colonies he found only 2 typhoid-like forms.

Isolation of the Cholera spirillum. The isolation of the cholera spirillum from water can probably be accomplished with somewhat less difficulty than is encountered in the case of B. typhi. Schottelius (Schottelius, 1885) was the first to point out the necessity for growing this organism in an alkaline medium, and Loeffler (Loeffler, 1893) found that its isolation from water could be successfully accomplished by adding 10 c.c. of alkaline pepton broth to 200 c.c. of the infected water and incubating for 24 hours at 37 degrees, when the organism could be found at the surface of the medium.

Somewhat earlier than this Dunham (Dunham, 1887) had made a special study of the chemical reactions of

the cholera spirillum and found that the organism would grow abundantly in a solution containing 1 per cent peptone and 0.5 per cent salt (Dunham's solution), producing the "cholera-red or nitroso-indol reaction." This medium was brought into practical use by Dunbar (Dunbar, 1892), who succeeded in isolating the organisms from the water of the Elbe in 1892, during the cholera epidemic at Hamburg.

Koch (Koch, 1893) prescribed the following method for the isolation of the organism from water:

To 100 c.c. of the water to be examined is added 1 per cent pepton and 1 per cent salt. The mixture is then incubated at 37 degrees. After intervals of 10, 15, and 20 hours the solution is examined microscopically for comma-shaped organisms, and agar plate cultures are made which are likewise incubated at 37 degrees. If any colonies showing the characteristic appearance of the cholera spirillum are found, these are examined microscopically, and if comma-shaped organisms are present, inoculations are made into fresh tubes to be further tested by means of the indol reaction and by inoculation into animals.

Stokes and Hachtel (1912) have suggested the use of a modified Hesse agar containing starch for the isolation of the cholera spirilla, which produce acid on such a medium, while the colon-typhoid organisms do not. The glycerin and lactose are omitted from the medium described on p. 78*c* and 10 gms. of soluble starch are added. The intestinal spirilla as a class form round, spreading, pinkish colonies on the starch medium, while colonies of other intestinal bacilli remain

blue. The medium is best used after the Koch enrichment method described above.

Other pathogenic organisms have been isolated from waters, according to the accounts of numerous investigators, but from the sanitary point of view the typhoid and cholera bacilli are of most importance, since these are manifestly the germs of disease most likely to be disseminated through this medium. For the detection of B. anthracis and other spore-forming pathogenic bacteria which may at times gain access to water from stockyards, slaughter-houses, etc., the method suggested by Frankland (Frankland, 1894) may be adopted. The water to be examined is heated to 90 degrees for 2 minutes and then plated, the characteristic colonies of the anthrax organism being much more easily discerned after the destruction of the numerous non-sporing water bacteria.

CHAPTER VI

THE COLON GROUP OF BACILLI AND METHODS FOR THEIR ISOLATION

The Colon Group of Bacilli. The Bacillus coli was first isolated by Escherich (Escherich, 1884) from the fæces of a cholera patient. It was subsequently found to be a normal inhabitant of the intestinal tract of man and many other animals, and to occur regularly in their excreta, and on this account it became of the highest interest and importance to sanitarians, since its presence in water-supplies was regarded as direct evidence of sewage pollution.

Specific disease germs are difficult to isolate even when they are present; and water may of course be grossly polluted with sewage without any specific disease germs being there at all. All sewage-polluted water, however, is potentially dangerous, since where fæcal matter exists, disease germs are at any time likely to appear. A test for fæcal material as distinguished from infected material is, therefore, essential; and for such a test the colon group of bacilli are specially well suited. They are not dangerous in themselves, but they are significant as indices of the probable presence of disease germs.

The so-called Bacillus coli may be described as a short,

usually motile rod, with diameter generally less than one micron and exhibiting no spore formation. It often appears in pairs of rods so short as to suggest a diplococcus. It decolorizes by the Gram stain. It forms thin, irregular translucent films upon the surface of gelatin, called "grape-leaf colonies" by the Germans, produces no liquefaction, and gives a wire-nail-like growth in stick cultures. It forms a white translucent layer of characteristic appearance upon agar, produces a more or less abundant, moist, yellowish growth on potato, and turbidity and some sediment in broth; it ferments dextrose and lactose with the formation of gas of which the ratio is approximately, $\frac{H}{CO_2} = \frac{2}{1}$, as ordinarily determined; a strong acid reaction and gas are produced in many other sugar-containing media. The organism generally gives a characteristic reaction in esculin media and typically reduces neutral red, changing its color to canary yellow with a greenish fluorescence. It grows in the Capaldi-Proskauer media, forming acid in the albumin-free medium, No. 1, and giving a neutral or alkaline reaction in the peptone-mannite medium No. 2. It coagulates casein in litmus milk, and reduces the litmus with subsequent slow return of the color (red), and generally forms indol in peptone solution. Many cultures of this organism are fatal to guinea pigs when the latter are inoculated subcutaneously with one-half c.c. of a 24-hour bouillon culture, and most cultures produce death when this amount is inoculated intraperitoneally. Although not a spore-forming bacillus, and in general not possessing

great resistance against antiseptic substances, B. coli is less susceptible to phenol than are many other forms, especially certain water-bacteria.

We have spoken as if Bacillus coli were a single definite organism. As a matter of fact it is a name applied to a considerable group of distinct forms which may be split up almost as far as one wishes by the application of various biochemical tests. The " colon bacillus," as we have pointed out, usually does not liquefy gelatin and reduces neutral red and coagulates milk, and produces indol; but there are closely allied forms which differ from the type in one or more of these respects. The colon group, as Smith (1893) long ago pointed out, may first be divided into two distinct subtypes according to the action of the organisms upon saccharose. One subtype forms gas and acid in saccharose media and the other does not. Winslow and Walker (1907) have found that those strains which ferment saccharose attack raffinose also, and point out that these two sugars which behave alike are those which lack the aldehyde grouping characteristic of dextrose and lactose. The application of tests in other carbohydrate media, such as dulcite, adonite, inulin, etc., make it possible to recognize perhaps a hundred distinct types each characterized by a particular combination of reactions.

The results obtained by the " colon test " will of course depend largely upon the definition of what a colon bacillus is; and there is marked disagreement upon this point among different observers. Konrich (1910) tabulates the tests used by 34 different workers, All of them defined the colon bacillus as a Gram-

negative non-spore-forming rod, but there was unanimity in no other respect; 26 of the 34 included the formation of acid and gas in dextrose media, 22 the coagulation of milk, 21 the formation of indol, 18 the formation of acid and gas in lactose media, and 18 the failure to liquefy gelatin. No other test was used by more than 13 out of the 34 observers. Konrich (1910) himself found that of over 600 colon-like organisms from fæces, all produced gas in dextrose broth, 79 per cent formed acid and 77 per cent gas in lactose broth, 65 per cent coagulated milk, 59 per cent fermented dextrose at 46°, 54 per cent reduced neutral red, 38 per cent formed indol.

Ferreira, Horta and Paredes (1908[a]) studied 117 strains of lactose-fermenting bacilli from human fæces. All proved to be motile and Gram negative, all coagulated milk and produced fluorescence in neutral red media, none liquefied gelatin in 15 days, all but one formed indol. Dextrose, lactose, maltose, galactose, and mannit were fermented by almost all strains, while gas was formed in saccharose by 38 per cent, in dulcite by 69 per cent and in inulin by 12 per cent of the strains studied. The "Proskauer reaction" (apparently the Voges-Proskauer reaction, though it is not quite clearly stated) was positive only 8 times out of 117 strains in dextrose, only 7 times (out of 48 strains tested) in galactose, and not at all in dulcite or inulin; lactose and maltose, on the other hand, showed it in almost every case. Copeland and Hoover (1911) report that out of 3000 colon-like organisms fermenting lactose bile 65 per cent gave positive tests for B. coli,

in milk, nitrate solution, pepton solution and gelatin; 28 per cent failed to produce indol and 5 per cent did not reduce nitrates. Numerous other results indicating similar variations are cited in Chapters VII and VIII.

Where shall the line be drawn? The English bacteriologists usually require in addition to the morphological characters mentioned above, motility, non-liquefaction of gelatin, fermentation of dextrose and lactose media, coagulation of milk, production of indol, and reduction of neutral red. The usual American procedure has included reactions in dextrose broth, milk, peptone solution (for indol), gelatin (absence of liquefaction), and reduction of nitrates. Of late years, however, there has been a growing feeling that such arbitrary definitions went either too far or not far enough. The whole group of lactose-fermenting bacilli is characteristically of intestinal origin. That we believe to have been clearly established by results to be cited later in this chapter and in the succeeding one. A differentiation between various sub-types of this group can only be properly justified by the fact that some of them are less resistant in water than others, and hence are indicative of fresh and recent pollution. There is some evidence that such is the case which will be discussed in Chapter VIII, but whatever may be concluded from the somewhat conflicting opinions on this point it appears certain that, in temperate climates at least, the whole class of lactose fermenters should be absent from safe water supplies. As the Committee on Standard Methods of Water Analysis

(1912) wisely concludes, "The entire group is typical of the presence of fæcal matter when water or sewage examinations are to be considered." The Committee defines the group as a whole by the following characteristics: "Fermentation of dextrose and lactose with gas production, short bacillus with rounded ends, non-spore-forming, facultative anaerobe, gives positive test with esculin, grows at 20° on gelatin and at 37° on agar, non-liquefying in 14 days on gelatin. Gram-staining negative." This definition again, however, includes debatable elements. It seems to us very doubtful whether there is sufficient evidence to warrant making the esculin reaction a general criterion of the colon group; and bacteria which liquefy gelatin more or less slowly grade into otherwise identical non-liquefying forms by almost imperceptible degrees.

We hesitate to add another to the long list of arbitrary definitions of the much-defined colon bacillus; but it does seem to us important to get down to rock bottom. From this standpoint we believe that the colon group may be defined as including all aerobic non-spore-forming bacilli which produce acid and gas in dextrose and lactose media. For practical purposes the test may be further reduced to positive reactions in a lactose fermentation medium, growth on an aerobic agar streak, and microscopic examination, since almost all forms which ferment lactose ferment dextrose as well. The relative importance of the various subdivisions of this general group will be discussed in Chapter VIII.

Isolation of Colon Bacilli by Direct Plating. The Wurtz litmus-lactose-agar plate (Wurtz, 1892), as noted in

Chapter IV, furnishes one ready method for the isolation of B. coli from water, and it was used by Sedgwick and Mathews for the purpose as early as 1892 (Mathews, 1893). The process is based upon the fact already alluded to, that B. coli readily ferments lactose with the formation of acid. If, therefore, plates are made with agar containing both lactose and litmus, the colon colonies develop as red spots in a blue field. Since organisms other than B. coli (notably the streptococci) may also develop red colonies, it is necessary to examine them further. This is done by fishing from isolated colonies, replating and inoculating into other media for identification.

The plate method of isolation is recommended by the Committee on Standard Methods of Water Analysis (1912) for sewages and polluted waters, in which colon bacilli are present in 1 c.c. or less. They recommend that Petri dishes with porous covers be used and that incubation be carried out at 40° instead of 37°. For success in the use of this method it is necessary to get a sufficient dilution so that colonies may be well isolated, and to this end it is advisable that a number of different dilutions be employed, a series of plates being prepared from each. Under any conditions the detection of the colon bacillus is seriously hampered by the development of other forms. Certain observers have therefore added phenol to the agar medium, combining the effect of high temperature and an antiseptic to check the growth of water-bacteria. Copeland for this purpose added to his tubes .2 c.c. of a 2 per cent solution of phenol (Copeland, 1901). Chick (Chick,

1900) found that 1.33 parts of phenol in 1000 materially decreased the number of colon bacilli which would develop, while 1 part gave very satisfactory results, the plates showing pure cultures of B. coli. The addition of antiseptics in this way is always open to the objection that weaker strains may be killed and lost.

In Germany the Endo medium and the Conradi-Drigalski medium have been extensively used for the direct isolation of colon bacilli with excellent results. The composition and use of these media have been discussed in Chapter V. It does not appear that they have sufficient advantages to compensate for the difficulty in preparing them.

The Use of Preliminary Enrichment Media in the Isolation of Colon Bacilli. The test for the colon bacillus may be made more delicate by a preliminary cultivation of the sample in a liquid medium for 24 hours at 37°, thus greatly increasing the proportion of these organisms present before plating. As suggested in the classic researches of Theobald Smith (Smith, 1892), this method may be made approximately quantitative by the inoculation of a series of tubes with measured portions of the water. If, for example, of ten tubes inoculated each with $\frac{1}{100}$ of a cubic centimeter, four show B. coli, we may assume that some 40 of these organisms were present in the cubic centimeter. Irons (Irons, 1901), in a comparative study of various methods for the isolation of B. coli, showed that the preliminary enrichment frequently gave positive results when the results of the direct use of the agar plate were negative, and

concluded that "where the amount of B. coli is small and the colony count large, the lactose plate for plating water direct is inferior to the dextrose fermentation-tube." Gage came to a similar conclusion (Gage, 1902).

The medium most commonly used in the United States prior to 1906 for preliminary enrichment was ordinary broth to which 1.0 per cent of dextrose had been added, and the reaction brought to the neutral point. Into each of a number of fermentation-tubes of this medium a measured quantity of the water to be examined is inoculated, and the culture is incubated for 24 hours at 37.5° C. It used to be customary to incubate for 48 hours. Recent experience has, however, shown that a 24-hour period gives approximately the same results if the production of gas rather than any specified amount of gas is the criterion of a positive test. Longley and Baton (1907) found that of 1091 enrichment tubes giving positive tests after 48 hours only 173 showed no gas in 24 hours; of these latter only two contained B. coli. The advantage of saving a day is so great as to warrant the adoption of the shorter period. At the end of 24 hours at least, the tubes are examined for gas formation. If gas is found, a small amount of the culture should be added, after suitable dilution, to litmus lactose agar and plated.

With polluted waters it will be found advantageous to plate out on the first appearance of gas (4–8 hours). It has been shown by one of us (Prescott, 1902[b]) that a very rapid development of B. coli takes place in the first few hours after dextrose solutions are inoculated with intestinal material, and a nearly pure growth of

colon bacilli often results, while other bacteria multiply more slowly. With highly polluted waters gas formation will probably begin within 12 hours, but with fewer colon bacilli present the duration must be increased. If the period of incubation be too long continued, trouble in the subsequent steps of the isolation may be encountered because of overgrowths by the sewage streptococci, or other forms which check the growth of the colon bacilli in the later stages of fermentation and finally kill them out. Even with pure cultures of colon bacilli Clemesha (1912[b]) has shown that sugar-broth tubes may be almost sterile after 4 days.

When it is desired to examine samples larger than 10 c.c. it becomes necessary to modify the enrichment process by adding the nutrient material to the water instead of the reverse. For this purpose dextrose broth or phenol-dextrose broth (consisting of broth with 10 per cent dextrose, 5 per cent peptone, and .25 per cent phenol) may be added to the sample of water to be enriched as suggested by Gage (Gage, 1901). Generally 10 c.c. of the broth is added to 100 c.c. of the water. The sample is then incubated at 37° for 24 hours, and if at the end of that time growth has taken place, a cubic centimeter is inoculated into a dextrose tube.

Advantages and Disadvantages of the Dextrose Broth Fermentation Tube. Experience with the dextrose broth fermentation tube as a first step in the isolation of colon bacilli soon led to the conclusion that a fair idea of the sanitary quality of water could be obtained from the results of this test taken by themselves and without the further process of isolating

specific cultures. It appeared that a rather definite proportion of tubes showing a characteristic fermentation proved on further examination to contain bacilli of the colon group; and it was therefore suggested that the dextrose broth test alone might be used as a rapid "presumptive" test. The underlying principle of this method is that B. coli develops rapidly in dextrose broth with gas formation of from 25 to 70 per cent of the capacity of the closed arm of the fermentation tube. Of this gas approximately one-third is carbon dioxide and two-thirds hydrogen, that is, as the gas formula is generally expressed, $\frac{H}{CO_2} = \frac{2}{1}$.

In testing a water by this method a series of samples, in suitable dilution, .001, .01, .1, 1.0, or 10 c.c., are added directly to the dextrose-broth tubes and incubated for 24 hours at 37°.

On measurement of the gas, if the results above given are obtained, the reaction is considered typical. If the amount of gas is between 10 and 25 per cent or more than 70 per cent, or the percentage of carbon dioxide is greater than 40, the reaction is considered atypical. If no gas forms, or less than 10 per cent, the test is called negative.

In recent years, Irons (Irons, 1901) was perhaps the first to call attention to the value of this method, stating that "when the dextrose tube yields approximately 33 per cent of CO_2, Bacillus coli communis is almost invariably present." In the next year the reliability of the fermentation test as an indication of B. coli was worked out by Gage (Gage, 1902) as given in the table on p. 110:

	1 c.c.	100 c.c.
Number of samples tested	5172	1375
Number giving preliminary fermentation	1036	474
Per cent of latter proved to contain coli	70	71

Whipple (Whipple, 1903) examined a large number of surface-water supplies by this "presumptive test" and obtained striking results, shown in the following table. The waters are arranged in six groups according to the results of sanitary inspection, group I including waters collected from almost uninhabited watersheds, and group VI waters too much polluted to be safely used for domestic purposes.

PERCENTAGE OF SAMPLES OF WATERS OF VARIOUS SANITARY GRADES GIVING POSITIVE TESTS FOR B. COLI WHEN DIFFERENT AMOUNTS WERE EXAMINED

(WHIPPLE, 1903)

Group.	0.1 c.c.	1.0 c.c.	10 c.c.	100 c.c.	500 c.c.
I	0.0	3.5	20.8	50.0	50.0
II	5.0	7.3	15.0	60.0	60.0
III	0.0	7.0	50.0	50.0	60.0
IV	4.0	6.8	41.7	67.0	75.0
V	5.0	13.0	75.0	100.0	100.0
VI	5.0	20.2	75.0	80.0	100.0

In view of these results Whipple suggested the following provisional scheme of interpretation:

Sanitary Quality.	Presumptive Test for Bacillus Coli.				
	0.01 c.c.	0.1 c.c.	1.0 c.c.	10.0 c.c.	100 c.c.
Safe	o	o	o	o	+
Reasonably safe	o	o	o	+	+
Questionable	o	o	+	+	+
Probably unsafe	o	+	+	+	+
Unsafe	+	+	+	+	+

It is undoubtedly true that a negative presumptive test is generally obtained with unpolluted waters. For example, in a study previously cited, Winslow and Nibecker (1903) reported that of 775 dextrose-broth tubes inoculated from 259 unpolluted sources only 41 showed gas. On the other hand, it is equally true that in a large proportion of cases colon bacilli are isolated from positive dextrose-broth tubes. Longley and Baton (1907) in the examination of 3553 samples of Potomac water obtained positive tests 794 times, while B. coli was actually present 529 times; 67 per cent of the presumptive tests were therefore correct. Gage (1902), in the Massachusetts work cited above, found that 70 per cent of his fermented dextrose tubes contained B. coli.

The work of recent years has made it clear, however, that both the coincidence of negative presumptive tests with the absence of B. coli and the general coincidence of positive presumptive tests with the presence of B. coli, are open to disastrous exceptions.

The errors in the dextrose broth test are both positive and negative; it may lead to the inference that bacteria of the colon group are present when they are not, and it may fail to show them when they are really there. In the first place, with some waters, positive presumptive tests may be obtained when colon bacilli are not present. According to Clark and Gage (1903) there are 58 well-described species of bacteria which give the presumptive test in dextrose-broth, of which 23 are widely separated from the B. coli group. An unpublished investigation by Winslow and Phelps

indicates that the result of the dextrose broth test is markedly influenced by the factor of temperature. Their work consisted in the examination of 185 samples of water from 90 different sources, ponds, brooks, pools, wells and springs in five different States, Maine, New Hampshire, Massachusetts, Michigan and Virginia, at three different seasons of the year. All the waters examined were, as far as could be determined, free from specific pollution, although washings from roads or pastureland might have had access to some of them. Most of the sources were undoubtedly unpolluted and the examination of 119 samples for B. coli yielded only 12 positive results. The presumptive test, however, was obtained in a large proportion of the cases, and much more often in summer than in winter or spring, as indicated in the table below.

DEXTROSE BROTH FERMENTATION IN 185 SAMPLES OF NORMAL WATERS AT DIFFERENT SEASONS

(Winslow and Phelps)

Percentage of Positive Results

	Summer, 1906.	Winter.	Spring.	Summer, 1907.
Framingham, Mass.	87	62	23	57
Ann Arbor, Mich.	95	47	....	....
Exeter, N. H.	82	10	44	50
Richmond, Va.	....	14	14	....
Mt. Desert, Me.	95	....	....	....
All stations.	91	37	25	54

The Ann Arbor waters in this series included a number of driven wells and the Mt. Desert sources were mountain brooks and ponds of the highest sanitary quality.

Fromme (1910) in this connection reports the results of 673 colon tests made on the water of the Elbe during a period of a year and a half. We have calculated from his figures the average results for the winter months and the summer months in the table below. It is evident that typical colon bacilli are nearly twice as numerous in the cold weather (for reasons discussed in Chapter I) while organisms fermenting dextrose broth but proving not to be B. coli are absolutely more abundant and relatively much more abundant in summer.

GAS PRODUCERS AND B. COLI IN ELBE WATER
(After Fromme, 1910)

	Positive Dextrose Broth Tests.	B. Coli Isolations.	Per Cent of Dextrose Broth Tests Showing B. Coli.
October–March.....	415	363	87
April–September....	258	170	66

Phelps and Hammond (1909) cite a very interesting case of the same phenomenon in the case of a ground water. A deep well at a hospital in Trenton, N. J. was temporarily polluted from a leaking sewer and after the source of pollution had been removed the condition of the water was carefully studied for a period of two months. During the period between Sept. 10 and Oct. 12 (the pollution being removed on Sept. 19) of 107 dextrose-fermenting microbes isolated 40 failed to produce gas in lactose broth; during the period between Oct. 12 and Nov. 9, 52 out of 64 dextrose-fermenting microbes failed to give gas in lactose broth. All through the investigation organisms of low fer-

mentative power, many of them liquefying gelatin, were present, but their numbers relatively increased during the period after the original pollution had been removed.

Much valuable light is thrown upon the significance of these positive dextrose broth results in the absence of the colon group by the investigations of Clemesha (1912[a]). In a careful study of 46 samples of human fæces and 25 different samples of cow dung, including about 3500 different colonies isolated by various methods, only about 5 per cent belonged to the class fermenting dextrose but not lactose. In rivers and ground waters, on the other hand, this group made up 24 per cent of the colon-like organisms present, and in lakes with long storage, 58 per cent. The table below shows the relative increase of the lactose negative forms with the natural purification of rivers in four dry months following rain and also their relative increase in settled and filtered water as compared with the raw river water. The numbers dealt with are too small to give entirely uniform results, but the general trend is clear.

PERCENTAGE OF DEXTROSE-FERMENTING ORGANISMS FAILING TO FERMENT LACTOSE. CALCUTTA WATER SUPPLY

(Clemesha, 1912[a])

Month	Oct.	Nov.	Dec.	Jan.	Feb.
Condition of river	Heavy rain	Very muddy	Clearing	Clear	Clear
Raw river water (80 colonies)	20	27	68	48	79
Settled water (20 colonies)	50	16	85	71	99
Filtered water (60 colonies)	29	33	72	98	95

Clemesha confirmed these results by a long series of examinations of naturally and artificially polluted waters, all tending to show that with fresh pollution most of the dextrose fermenters ferment lactose as well, while with storage there is a relative increase in the lactose-negative forms. Careful studies of the history of fæcal mixtures in water showed that the resistant form was a particular type, called by Clemesha Bacillus P. Houston (1911), reports similar results for London waters. Of 12,744 specimens of raw river water containing dextrose-fermenting organisms 81 per cent gave positive results in lactose and formed indol as well, thus indicating the presence of the colon group. Of 18,960 specimens of filtered water containing dextrose-fermenting organisms only 51 per cent gave positive results in lactose and formed indol. Clemesha (1912[b]), in an analysis of Houston's results, shows that the preponderance of dextrose-positive lactose-negative forms is here not due to the Bacillus P, which Clemesha found in India, but to two different forms.

From all these investigations it is clear that the dextrose broth test does not bear a constant relation to the presence of the colon group, since another type of organisms fermenting dextrose but not lactose is relatively much more abundant in stored and relatively pure water, particularly in warm weather.

Phelps and Hammond (1909) have pointed out a rather serious error in the routine isolation of B. coli, as it used to be practised in this country, due to the presence of this group of organisms which fail to form

gas in lactose broth. The five standard tests for B. coli which have been most generally adopted in the United States included gas production in dextrose broth and coagulation of milk, but not gas production in lactose broth. It was supposed that types producing gas and acid in dextrose media and coagulating milk, but failing to form gas in lactose broth, would be rare. In the particular polluted well studied by Phelps and Hammond, however, such forms were very common, outnumbering true colon bacilli four to one during the latter part of the investigation when the pollution was less recent. Two workers following the standard methods but using dextrose broth for enrichment on the one hand and lactose broth on the other would in 50 per cent of the samples tested have reached opposite conclusions as to the presence or absence of B. coli, the isolations begun with dextrose broth being apparently positive and those begun with lactose broth being negative.

It is also clear on the other hand that the dextrose test as ordinarily used may be negative when colon bacilli are present. This is due to the interaction of various bacteria in the fermentation tube and to the solution and escape of gases which often prevents the production of the typical gas formula. Of 43 cultures isolated by Fuller and Ferguson (1905) at Indianapolis, 18 showed less than 20 per cent of gas after 48 hours in the enrichment tube, and 11 showed less than 10 per cent. Hale and Melia (1910), working with a pure culture of B. coli in unsterilized water (containing no other gas former) report that of 818 tubes showing gas

only 474 or 58 per cent gave between 25 and 70 per cent of gas in the closed arm with 25–40 per cent carbon dioxide.

Stamm (1906) and others have pointed out that the ratio of carbon dioxide to hydrogen changes with the age of the culture. At first the proportion of the former to the latter is as two to one, and later, in the same tube, the ratio is reversed. More recently, Longley and Baton (1907), in one of the ablest and most fruitful of recent contributions to water bacteriology, have made it clear that neither of these quantitative determinations is of importance if made in an ordinary open tube. They show, first, that the total amount of gas formed by B. coli varies widely, from 10 to 80 per cent, the mode of the curve being found, not at 50, but at 35 per cent. Secondly, they show that the proportion of carbon dioxide present is a function of the total amount of gas. They find that when grown in an atmosphere of CO_2, B. coli produces a gas which consists of about 3 parts of carbon dioxide to one of hydrogen. Assuming that the gas originally formed by B. coli has always about this composition, and that the absorption of CO_2 by the medium is the chief cause of the differences observed in the gas which collects in the closed arm, the gas ratio would vary directly with the amount of total gas; the more rapidly gas is formed, the greater the proportion of CO_2 remaining unabsorbed. Calculation on this basis gives a curve very close to the observed data.

These criticisms apply only to the fermentation test made in an open tube and uncorrected for the

absorption of CO_2. Keyes (1909) and others have introduced more exact methods based on the collection and analysis of all gases formed in a vacuum, and Rogers, Clark and Davis (Journal of Infectious Diseases, 1914, XIV, 411) in an important paper which we discuss more fully on page 200, have shown that the gas ratio when accurately determined is highly characteristic for certain members of the colon group.

Not only is it true that little reliance can be placed on the exact gas formula in the open dextrose broth tube, but cultures of the colon group may be actually overgrown and lost by the multiplication of other forms. This is particularly true when the waters are heavily polluted or when large samples are examined. Hunnewell and one of us (Winslow and Hunnewell, 1902[b]) found that of 48 samples of certain polluted river waters 18 showed B. coli when 1 c.c. was inoculated directly into dextrose broth, while in only 4 cases was a positive result obtained after preliminary treatment of 100 c.c. in carbol broth. In 153 samples from presumably unpolluted water B. coli was found 5 times in 1 c.c. and 11 times by the examination of the larger sample. It will be noted that these results were obtained with carbol broth for the enrichment of the larger samples and carbol broth is less liable to overgrowths than dextrose broth.

Whipple (Whipple, 1903) notes that 2.9 per cent of some samples of water examined by him gave positive tests with .1 c.c. but not with 1 c.c., while 4.3 per cent gave positive tests with .1 c.c. or 1 c.c. and negative

tests with 10 c.c. Again, in another series of samples examined, of those which gave positive tests in smaller portions 5.3 per cent were negative in 10 c.c., 4.7 per cent in 100 c.c., and 7.7 per cent in 500 c.c.

Fromme (1910) has made an interesting study of this point and reports that of 59 samples of water of good quality which showed B. coli in small portions 25 per cent gave negative results in larger portions; while of 654 samples of polluted waters 33 per cent gave negative results in large portions and positive results in smaller ones. These results are of value as indicating the greater liability to loss by overgrowth in polluted waters; but the absolute figures are much higher than workers in this country obtain when the enrichment cultures are carefully watched and platings made from them at an early period.

The Use of Phenol Broth as an Enrichment Medium to Check Overgrowths. As has already been stated, phenol has less inhibitory action upon B. coli than upon normal water-bacteria, and it was hoped that a broth containing this substance might be employed for preliminary enrichment with advantage, its inhibitory power checking the overgrowing forms, but not B. coli. This medium was used in place of dextrose broth for many of the studies made in connection with the Chicago drainage canal (Reynolds, 1902). Phenol broth consists of ordinary broth to which 0.1 per cent phenol is added, and the method of procedure is to add 1 c.c. of the water to 10 c.c. of the sterilized phenol broth and incubate at body temperature for 24 hours. Litmus-lactose-agar plates are then made and the examination

of the red colonies carried out as described for the dextrose-broth method. It has unfortunately proved, however, that with waters of fairly good quality the phenol interferes with the colon bacilli themselves to a serious extent. The dextrose broth furnishes a more delicate test than the carbol broth when the number of colon bacilli present is small, as is clearly shown by the following table from Irons:

PROPORTION OF POSITIVE RESULTS IN TESTS OF POLLUTED AND UNPOLLUTED WATERS BY DEXTROSE FERMENTATION-TUBE AND CARBOL-BROTH METHODS

(IRONS, 1901)

	Dextrose Fermentation-tube.			Carbol-broth Method.		
	+	−	?	+	−	?
Polluted waters	33	31	5	38	30	1
Relatively unpolluted waters	56	38	25	37	61	21

The Eijkman Test. Another enrichment test, which has been extensively used in Germany for checking the development of overgrowing forms and limiting the bacteria in the fermentation tube to the colon group, is the Eijkman test, which depends on the use of a high temperature (46°) (Eijkman, 1904). There is no doubt that such a procedure cuts out the water bacteria, and Christian (1905), Neumann (1906), and Thomann (1907) have reported good results from its use. Hilgermann (1909), too, urges the value of the Eijkman test and concludes that the colon-like bacilli, which fail to grow at 46°, are characteristic of comparatively unpolluted waters. Other observers maintain,

and apparently with good reason, that the conditions of this test are too severe and eliminate many intestinal bacilli of undoubted significance. Nowack (1907) found that laboratory cultures of B. coli often fail to produce gas in Eijkman's medium at 46°, unless large numbers are introduced. With some strains an inoculation of over a million bacteria was necessary to cause gas formation.

Konrich (1910) compared the Eijkman enrichment method (dextrose peptone water at 46°) and that of MacConkey (dextrose-bile-salt peptone water at 42°) with 57 water samples and obtained only about 70 per cent as many positive results with the former as with the latter method. With artificial emulsions of pure cultures and of fæces even greater differences were manifest. These studies showed conclusively that incubation at 46° prevents the development of great numbers of colon bacilli and is unsuitable for an enrichment process. In comparing the two media (dextrose peptone water with and without bile salts) at the same temperature, 37°, he obtained essentially similar results.

Fromme (1910) has shown that during the first 5 hours in various enrichment media colon bacilli multiply more rapidly at 46° than at 37°. After that time, however, their development is checked. At 12 hours the numbers at the two temperatures are about equal and between 12 and 24 hours the numbers increase much more rapidly at 37°.

The Neutral Red Reaction. Other special media have been suggested for rapid routine water analysis,

of which those containing "neutral red," one of the safranine dyes, have been somewhat fully studied. Rothberger (Rothberger, 1898) first pointed out that B. coli reduces solutions of this substance, the color changing to canary-yellow accompanied by green fluorescence. Makgill (Makgill, 1901), Savage (Savage, 1901), and other English observers, as well as Braun (1906), in France, report favorable results from the use of this test; but according to American standards, Irons (Irons, 1902) and Gage and Phelps (Gage and Phelps, 1903) conclude that the group of organisms giving a positive neutral red reaction is too large a one to give very valuable sanitary information.

Stokes (1904) urged the use of lactose broth with the addition of neutral red, and believed that the production in this medium of 30-50 per cent of gas with a $\frac{2}{1}$ gas formula and the change of neutral red to canary yellow in the closed arm of the fermentation tube was characteristic for B. coli.

The Lactose Bile Test for the Colon Group. On the whole, by far the most satisfactory results in making a rapid test for the colon group have been obtained by the use of media containing bile salts, a procedure the development of which in this country we owe principally to Jackson (1906).

MacConkey (1900) long ago suggested the use of media containing bile salts (sodium taurocholate) for the differentiation of B. coli and B. typhi, and bile-salts media have been used by various English observers (MacConkey, 1901; MacConkey and Hill, 1901) for

the isolation of sewage bacteria. Jackson studied the action of various bile media and showed their selective inhibitory action in the striking table quoted below. His important contribution to the subject, however, was the discovery that ox bile itself could be used as a culture medium, and that it was easier to prepare, cheaper and more effective than combinations of meat infusion with the purified bile salts.

SELECTIVE ACTION OF BILE SALTS

(JACKSON, 1906)

	Bacteria per c.c.			
	Uncontaminated Well.	Contaminated Pond.	Suspension of Fæces.	Suspension of Fæces.
Gelatin, 20°	920	2700	350,000	900,000
Agar, 37°	25	170	450,000	900,000
Bile agar,* 37°	14	43	300,000	900,000
Lactose bile agar,* 37°	0	25	250,000	675,000
Lactose bile agar,* 37°	0	17	250,000	600,000
Bile agar, 37°	0	16	60,000	900,000

* Bile diluted, 1 : 1.

Jackson suggested the use of fresh ox bile containing 1 per cent of lactose as a presumptive test instead of dextrose broth. In particular he hoped that this medium would be free to a great degree from the negative results due to overgrowths in polluted waters. He reported 275 examinations of badly contaminated waters, in which 65 per cent of the samples failed to give the dextrose-presumptive test, and only 10 per cent failed to show gas in lactose bile. In a more recent communication, Jackson (1907) reports that in the examination of

5000 samples of water at the Mt. Prospect Laboratory, the bile medium has proved uniformly satisfactory. He recommends incubation for 72 hours, results being commonly obtained, however, after 48 hours; and he considers any tube showing 25 per cent gas as positive. In a series of examinations carried out at the Institute of Technology, 16 per cent of the positive tubes showed

COMPARATIVE PRESUMPTIVE TESTS WITH DEXTROSE BROTH AND LACTOSE BILE

(SAWIN, 1907)

	Source.	Percentage of Samples Giving Positive Tests for B. Coli.					
		Dextrose Broth.			Lactose Bile.		
		0.1 c.c.	1.0 c.c.	10.0 c.c.	0.1 c.c.	1.0 c.c.	10.0 c.c.
1	Deep wells	0	0	0	0	0	0
2	Shallow wells	0	1.0	10.0	0	0	6.0
3	Lake	15.0	10.0	15.0	5.0	0	15.0
4	Lake	5.2	15.7	21.0	5.2	5.2	31.0
5	Lake	10.0	5.0	40.0	10.0	5.0	15.0
6	Lake	10.0	10.5	26.0	10.0	10.5	50.0
7	Lake	0	10.0	5.0	0	0	15.0
8	Lake	10.0	15.0	35.0	0	5.0	30.0
	Average, Nos. 3, 4, 5, 6, 7, 8	8.3	11.0	23.6	5.0	4.3	26.0
9	River	47.0	72.2	55.5	50.0	75.0	84.2
10	River	26.3	37.6	73.7	30.0	90.0	85.0
11	River	36.8	55.1	68.9	40.0	73.5	78.1
	Average, Nos. 9, 10, 11	36.7	55.1	66.0	40.0	79.4	82.4
12	Brook	47.7	63.2	72.2	60.0	90.0	84.2
13	Drainage	50.0	73.7	78.9	84.2	90.0	90.0
14	Sewage	25.0	25.0	8.2	87.5	93.7	81.2
	Average, Nos. 12, 13, 14	40.9	53.9	53.1	77.2	91.2	85.1

gas in 24 hours, 73 per cent after 48 hours, and the remaining 27 per cent only after 72 hours; but the Committee on Standard Methods (1912) believes that the forms which fail to develop in 48 hours are attenuated forms of little practical significance. Sawin (1907) reports comparative results with dextrose broth and bile on different classes of waters, the most striking of which are tabulated on p. 124.

Like other enrichment methods which eliminate competing forms it is no doubt true that the lactose bile test cuts out some weak colon bacilli. As a presumptive method, however, it is far superior to dextrose broth, giving a higher proportion of positive tests with polluted waters and a lower proportion of erroneous positive tests with waters of good quality. In an examination of 176 surface waters in eastern Massachusetts, carried out under our direction, B. coli was isolated 70 times. The dextrose-broth test was positive 120 times, an error of 70 per cent; while the bile test, alone, was positive 78 times, an error of only 11 per cent. The tabulated results of these experiments indicates fairly the merits of the bile medium for preliminary enrichment and as a presumptive test.

PRELIMINARY AND COMPLETE RESULTS OF DEXTROSE BROTH AND BILE TESTS. 176 SURFACE-WATERS

	Preliminary Positive Results. (Gas Formation.)	Final Positive Results. (B. Coli).
Dextrose broth.....	120	70
Lactose bile.....	78	64

Hale and Melia (1910) have also made a valuable comparison of a number of presumptive tests as applied during a period of 2 years to 85 samples of Manhattan water (surface) and 160 samples of Brooklyn water (largely ground-water). Their principal results are tabulated below.

RESULTS OF VARIOUS PRESUMPTIVE TESTS

(HALE AND MELIA, 1910)

245 Samples of New York Water

Medium.	Percentage of Positive Results in		
	0.1 c.c.	1.0 c.c.	10.0 c.c.
Dextrose broth (standard gas formula) ...	0.4	7.0	30.7
Lactose bile	0.8	8.2	38.4
Lactose-peptone bile	0.8	15.5	51.2
Dextrose broth, all tubes showing 5% gas transplanted to bile	0.8	12.0	57.6
Dextrose broth, 5% gas and over, called positive	6.3	31.4	73.5

This table indicates very clearly the fallacies of the dextrose broth tube. Counting all gas formers in this medium as positive indicates much too high a value and including only the tubes showing the standard gas formula gives much too low a value. This conclusion is based on the assumption, warranted by the results of many workers, that incubation in dextrose broth followed by reinoculation into lactose bile (fourth line of the table) gives with reasonable accuracy the real number of colon bacilli present. By this standard the use of plain lactose bile with these waters is seen to give results which are also much too low; but lactose peptone bile approximates closely to the truth.

Of course it must be remembered that the advantages of lactose-bile over dextrose broth are partly due to the inhibiting effect of the bile salts and partly to the use of lactose instead of dextrose which cuts out the dextrose-positive lactose-negative group to which allusion has been made earlier in the chapter. The relative importance of these two factors, lactose and bile, is well brought out in a study by Stokes and Stoner (1909). These authors have compared a considerable series of preliminary enrichment tests followed by final isolation in dextrose broth, lactose broth and lactose bile. Of 567 colonies from positive dextrose broth tubes only 52 per cent were colon bacilli; of 3752 colonies from positive lactose broth and lactose bile tubes, 88 per cent of the lactose broth colonies and 95 per cent of the lactose bile colonies were B. coli.

With sewages and heavily polluted waters in particular the lactose-bile medium has proved of the greatest value. When a large proportion of sewage is present the colon bacilli are fresh from the intestine and apparently able to resist the antiseptic salts. On the other hand, the high numbers of other bacteria present make the danger of overgrowths particularly great. With waters of fair quality, such as those with which we ordinarily deal in sanitary water analysis, lactose bile is open to the same objection as phenol broth and the Eijkman test though in less degree. It inhibits not only the overgrowing forms but the weaker representatives of the B. coli group itself, and the net effect is to diminish positive results.

Hale and Melia (1910) inoculated unsterilized water

(shown to contain no gas formers) with a pure culture of B. coli and stored it for different periods and under different conditions, testing at intervals by various presumptive tests. The colon bacilli lived for 8–10 days at 37°, for 38–75 days at 20°, and 77–84 days at 8° C. Comparison of presumptive tests with plate counts on litmus-lactose agar showed that a gas test in dextrose broth corresponded to an average of 4 bacteria and a positive test in lactose bile to 39 bacteria. In general the dextrose broth showed gas in one dilution higher than the lactose bile; and the difference increased with the attenuation due to prolonged sojourn in water.

These results of Hale and Melia make it clear that the selective action of bile salts upon the various members of the colon group may perhaps be an advantage rather than a disadvantage for practical sanitary purposes. The Committee on Standard Methods (1912) recognizes that lactose bile does inhibit certain weaker members of the colon group, but believes that these attenuated organisms indicate only remote pollution and are of little significance. They say, "In the interpretation of the sanitary quality of a water, it is best to discount the presence of attenuated B. coli and to be sure to obtain all vigorous types. The lactose bile medium accomplishes both of these objects."

The advantages of the dextrose broth enrichment of weak colon bacilli and of the elimination of gas-forming organisms other than B. coli by bile may both be obtained as pointed out above by inoculating lactose bile from

the dextrose broth. Hale and Melia (1910) find that this gives results in as high a dilution as by the use of dextrose broth and with the clear-cut results of lactose bile.

The Aesculin Test. A test for the colon group which has attracted much interest during the last few years is the fermentation of the glucoside aesculin. B. coli effects a hydrolytic splitting of this substance, producing sugar and a substance called aesculetin, which reacts with iron citrate to produce a dark brown salt. Harrison and van der Leck (1909) have used broth or agar made up with 1 or 2 per cent Witte's peptone, .5 per cent sodium taurocholate, .1 per cent aesculin and .05 per cent citrate, and find that the black colonies with a black halo produced by the colon group of organisms are highly characteristic. Of 60 samples of water which showed blackening of aesculin broth, all proved to contain B. coli. Hale and Melia (1911) have shown that two species of streptococci, the aurococcus, and the bacillus of pneumonia, also give the aesculin reaction and that in the absence of the bile salt which the aesculin medium contains a number of other forms may ferment this glucoside.

On the whole we do not think it has been shown that aesculin has sufficient differential value to warrant its inclusion in enrichment media to be used in the colon test. It appears that the anaerobic B. welchii is practically the only form outside the colon group which produces a characteristic reaction in lactose bile and not in aesculin-bile salt media. It may prove

worth while to use aesculin to exclude this form, but the more general tendency at present seems to be to eliminate the anaërobic spore formers by subsequent subcultures.

The Use of Synthetic Media for the Isolation of the Colon Group. Dolt (1908), working in Prof. Gorham's laboratory at Brown University, has attempted with success to substitute synthetic media of simple and known composition for the usual meat-infusion-peptone media used in the isolation of B. coli. He first found that colon bacilli will grow readily on a medium containing asparagin and sodium or ammonium phosphate. He then attempted to substitute for asparagin various simple organic substances similar in their structure to the cholic acid of the bile which exerts a selective action in favor of the colon group. He finally succeeded in preparing two media which promise to be of considerable value in permitting the growth of B. coli while checking other forms. The first of these media is made up as follows: 500 c.c. of a 3 per cent solution of purified agar is mixed with an equal portion of a solution of 1 per cent glycerin and 0.2 per cent $(NH_4)_2HPO_4$. It is neutralized with sodium hydroxide and 1 per cent of lactose is added before sterilization. In the second medium 5 gm. of ammonium lactate is substituted for the glycerin and 1 gm. of Na_2HPO_4 for the ammonium phosphate. These media proved to have a considerable selective value, cutting out most of the water bacteria; but like all such selective media they cut out a good many of the colon bacilli too. The results of a single test are shown in the table below. The procedure well merits further study, however.

COMPARATIVE RESULTS OF ORDINARY AND SYNTHETIC AGAR MEDIA

(Dolt, 1908)

	Total Colonies.	Red Colonies.	B. Coli.
Standard agar.....	67	38	18
Glycerin agar.....	27	9	9
Ammonium lactate agar	17	9	9

The Use of Liver Broth for the Isolation of a Maximum Proportion of Gas-forming Bacteria. The media we have been discussing, phenol broth, dextrose broth incubated at 46°, and bile, are designed to cut down the gas producers which appear in ordinary dextrose broth so that only vigorous typical members of the colon group are able to develop. For special research purposes when it is desired to get the largest possible proportion of gas formers of all kinds, there are other media which give even more positive fermentation results than dextrose broth itself. The most important of these is the liver broth of Jackson and Muer (1911) made up with beef liver, peptone, dextrose and potassium-acid-phosphate. The Committee on Standard Methods of Water Analysis (1912) recommends that if "a study of all gas-forming bacteria, including attenuated forms, is desirable, then liver broth should be employed in preference to the usual dextrose broth, as it gives a larger number of attenuated forms, has better rejuvenating power, and gives fewer anomalies and greater and more rapid gas production." In order to avoid attenuation or inhibition transplants

should be made from this enrichment medium after 6–12 hours at 37°.

Isolation of Pure Cultures from the Enrichment Tube. In case one does not rely upon a "presumptive" test alone but desires to study the organisms present in detail the isolation upon a solid medium, usually litmus-lactose-agar in this country, must follow the enrichment process. Since the enrichment tube was inoculated with a known amount of water all further work is purely qualitative, and it is only necessary to obtain such a number of colonies upon the lactose plate that the isolation of a pure culture shall be easy. In practice the following procedure has been found generally successful. After the enrichment tubes have been incubated for 12 to 24 hours at 37°, from those which show gas, one loopful is carried over to a tube containing 10 c.c. of sterile water, and of this water one loopful is taken for the inoculation of the plate. Ordinarily this will give colonies which are sufficiently well separated, but a second plate, inoculated from the dilution water with a straight needle instead of a loop, furnishes a desirable safeguard. With practice it is possible to effect a proper seeding more rapidly by barely touching the tip of a straight needle to the broth in the fermentation tube and transferring this directly to the agar. The touch must be a very light one, however, or the colonies on the plate will be too thick for proper isolation.

The litmus-lactose-agar plates made in this manner should be incubated for from 12 to 24 hours at the body temperature (37°), at the end of which time, if B. coli is

present, red colonies upon a blue field will be visible. The litmus-lactose-agar plate may become blue again after 48 hours, owing, presumably, to the formation of amines and ammonia by the action of the bacteria upon the nitrogenous matter present. If the dilution is too low, the resulting colonies will be small and imperfectly developed, making it difficult to be sure of pure cultures for the subsequent tests. A great number of colonies will also prevent the change of reaction from acid back to alkaline.

In the selection of those red colonies which are to be fished from the litmus-lactose-agar plate the appearance of the growths must be closely noted. A colony of irregular contour, surrounded by a very faint area of reddening, will probably belong to some member of the B. mycoides group (Winslow and Nibecker, 1903); small, compact, bright-red colonies are characteristic of the streptococci, and Gage and Phelps (Gage and Phelps, 1903) have pointed out that of these there are two types, one of a brick-red color, and of such consistency as to be readily picked up by the needle-point, and the other smaller and of an intense vermilion color. The colonies of the colon bacillus are usually well formed, pulvinate on the surface and fusiform when growing deeper down.

If no red colonies appear on the litmus-lactose-agar plate after a positive result in dextrose broth one of four things has occurred: There may be an organism present which forms gas in dextrose but no acid in lactose; there may be present forms which individually fail to attack lactose but growing together, symbiotically,

produce gas in dextrose; B. welchii or some other form which will not grow on aerobic plates may have produced the gas; or an organism orginally present and capable of fermenting both sugars may have been overgrown and lost in the enrichment tube. If plates are made on the first appearance of gas the likelihood of the latter possibility will be reduced to a minimum. Neither of the first two contingencies has any sanitary significance; as we have seen, bacteria which ferment dextrose and not lactose are not specially characteristic of pollution. In general, therefore, the absence of red colonies on the agar plate may be considered a negative result. If red colonies are present they must be subcultured and examined further.

The agar streak made from the litmus-lactose-agar plate shows after 24 hours certain marked characteristics. The most distinct types are two, the abundant, first translucent, later whitish and cheesy growth, covering nearly the whole surface of the agar, characteristic of B. coli and its allies, and a very faint growth, either confined strictly to the streak or made up of faint isolated colonies, dotted here and there over the surface. The latter cultures are typical of the sewage streptococci, and a microscopic examination will generally settle their status at once. Of the more luxuriant growths, some of which are stringy to the needle, many will generally prove to be atypical, and if any of the weakly fermenting forms (B. mycoides) are present a dull wrinkled growth will be produced.

The various tests which may be applied to the cultures after they have been isolated, the subgroups

into which the colon group may be divided by their use, and the significance of the results obtained will be discussed in Chapter VIII.

Practical Routine Test for the Colon Group. As has been pointed out above the aggregation of lactose-fermenting bacilli which we call the colon group may be almost indefinitely subdivided by the application of a more or less elaborate series of diagnostic tests. Each observer in the past drew up a scheme of what he believed to be essential tests and called all the bacteria which failed to conform to them "atypical." The more of such "atypical" forms a particular worker includes the greater will be the number of positive isolations. The definition of this or any other bacterial species is more or less arbitrary; we consider as true colon bacilli those which fulfil a particular set of tests, and class as pseudo-colon organisms those which do not. If we find, having established such an arbitrary standard, that the colon bacillus, as determined by it, is found in waters known to be polluted, and not, as a rule, in those known to be free from pollution, the sanitarian can afford to ignore the theoretical question of specific values and make confident use of the practical test. In order that results may rest on a sound basis of comparable data for various waters, it is of course essential, however, that a standard set of reactions should be agreed upon by sanitary bacteriologists.

After a considerable period of uncertainty, in which each observer used the procedure which happened to appeal to him, the attainment of comparative results was made possible by the establishment of standard

methods of procedure by bodies of authoritative position, both in England and America. In 1904 an English Committee, appointed to consider the Standardization of Methods for the Bacterioscopic Examination of Water, presented a series of obligatory tests and optional tests; and in 1905 the Committee on Standard Methods of Water Analysis of the American Public Health Association drew up a set of diagnostic characters for B. coli. The latter corresponded in general with the plan developed by the Massachusetts State Board of Health (Massachusetts State Board of Health, 1899) and long in use at the Massachusetts Institute of Technology, and involved the determination of morphology, motility, fermentation of dextrose broth, coagulation of milk, production of indol and reduction of nitrates. The English standard procedure corresponded quite closely to this (Committee appointed to consider the Standardization of Methods for the Bacterioscopic Examination of Water, 1904), although it differed from the American method in certain respects.

Since these standards were formulated 8 years ago their artificial nature has been made more and more manifest to those who have used them. A still more fundamental question, however, has pressed itself upon the practical analyst. Each lactose bile presumptive test involves the use of a single tube of medium and the work is complete in 48 hours. The "complete" test as used in America required the use of seven different media and took 9 days to complete, since the gelatin subculture must be incubated for

at least a week. Granting that the lactose bile test gave us the whole colon group and that the " typical B. coli " giving characteristic reactions in milk, peptone, gelatin and the nitrate solution, only the more sensitive members of the group, indicative of recent pollution, was the extra information gained worth the additional trouble? Under the conditions which generally obtain, in the United States at least, it appears not. The colon test at best is an approximate one, and its results are usually only expressed in decimal fractions, positive in 10 c.c., 1 c.c., or .1 c.c. for example. From 70 to 90 per cent of the bacteria which give the lactose bile test prove to be " typical " B. coli on any of the definitions ordinarily used. This makes a difference so slight as to be almost negligible. We cannot condemn a water because it contains 10 rather than 7 colon bacilli in a given proportion. It may be that under tropical conditions such as those described by Clemesha (to be discussed in a following chapter) certain forms of the colon group persist for a long time in stored waters from which disease germs have disappeared. These resistant forms must, however, be studied by a much more elaborate procedure than the 7 tests in the old American Standard method; and it seems clear that they do not occur in large numbers in temperate climates. For our conditions the whole group of forms which produce gas in lactose bile should be absent from safe waters.

The Committee on Standard Methods of Water Analysis in its last report (1912) apparently takes this ground, although its discussion of the problem

is somewhat ambiguous. In order to clear up the subject, the Laboratory Section of the American Public Health Association, at its Washington meeting, in September, 1912, adopted a resolution recommending determinations of counts at 20 degrees and 37 degrees, and the lactose bile presumptive test as the standard routine procedure in water examinations.

Recent Criticisms of Lactose Bile Test. During the last two years a number of workers have found that the presence of anaërobic bacilli of the B. sporogenes type, giving a misleading positive result in the lactose bile tube, may be a seriously confusing factor in interpreting the results of examinations of certain waters.

Mr. George W. Fuller has recently pointed out that at certain points on the Ohio and Mississippi Rivers the presumptive test yields results apparently far too high to be explained by ordinary pollution, and much higher than the results of a complete isolation test as shown at the same localities.

Phelps and his associates in the study of the Potomac have observed that in waters showing recent pollution in large amount, the presumptive tests in both lactose bile and lactose broth were efficient and reliable. As the pollution becomes less evident, that is, as purification proceeds, however, these media give increasingly unreliable results. A study of a large number of samples in which confirmatory tests were made showed that as the purification increased, the organisms appearing as fermentation exciters in the presumptive tests were more and more frequently not of the colon type, but instead proved to be organisms

of anaërobic character giving large amounts of gas, even up to 100 per cent, a milky appearance and a vile odor in lactose broth instead of the rather clean acid smell characteristic of B. coli.

Endo plates from these tubes were either atypical or showed no growth. These organisms are probably of the spore-forming B. sporogenes type, a group which we know from the work of Houston and others to be widely distributed. Wells found this organism in the water at Grand Forks, N. Dak., after treatment with calcium hypochlorite. They appear to grow in decomposing organic matter, deposited sludge from sewage, etc. The occurrence of this organism was noted by A. J. McLaughlin in the report of the Commission to investigate the waters of the International Boundary (Rivers and Lakes Commission).

The special significance of these facts is that when some waters originally highly polluted reach the final stages of purification these gas-forming anaërobes may appear and make the presumptive test less delicate and accurate. This seems to be especially the case in the examination of waters in connection with the shellfish industry.

This state of affairs is noted in the Potomac River below Washington, as will be seen by the following table kindly furnished by Mr. W. F. Wells. (See page 139*a*.)

It is very evident from columns 2 and 5 that the greater the number of B. coli the higher is the percentage efficiency of the presumptive test.

A number of observers have also recently emphasized

the fact (pointed out on page 127) that lactose bile is less sensitive than lactose broth as a presumptive test, as shown by the number of positive isolations of B. coli recorded. Thus Young, of the Kansas State Water Survey, notes that out of 140 samples showing fermentation in the presumptive test, 116 were found to give

Station.	No. of Samples.	Average B. coli.	Avg. Fermenting Organisms not B. coli	Percentage not B. coli to B. coli.	Percentage Fermenting Organisms Confirmed B. coli.
Giesboro Point.....	770	295	24.1	8.1	92.5
Fort Foote.....	789	254	9.7	3.8	96.4
Fort Washington.....	778	123	6.2	4.8	95.2
Mt. Vernon to Whitestone Point.....	851	102	8.5	8.3	92.2
Indian Head.....	241	123	15.7	12.8	88.6
Possum Point.....	212	66.2	13.8	20.8	82.4
Maryland Point.....	681	1.44	.75	52.1	68.9
Pope's Creek.....	740	.19	.33	174.0	36.6
Lower Cedar Point...	476	.14	.16	114.0	46.7
All below Lower Cedar Point.....	2261	.052	.057	110.0	47.7

positive confirmatory results for B. coli. Of these, 95 were positive in the lactose bile presumptive test, but 20 samples showed B. coli with lactose broth which were negative with lactose bile. In order to overcome these irregularities, a method of cross-inoculation was followed, using three media—lactose bile, lactose broth and dextrose bile. The procedure was as follows: Each sample of water was inoculated into the three media, using one large fermentation tube with 10 c.c. of the sample and five ordinary tubes with 1 c.c. each. Incubation for 24 hours was maintained. If the fer-

mentation with gas-formation had taken place in only one or two of the three varieties of media, cross-inoculation was made into the three media again and into dextrose broth. Streaks were also made on litmus lactose agar. Incubation was again maintained for 24 hours. It was found that organisms which would ferment all four media after cross-inoculation always proved to be B. coli, and only rarely was there any failure to confirm B. coli when all the original three media gave positive presumptive tests. These failures were ascribed to anaërobic organisms, but no isolations were made. They were so rare, however, as to be negligible, and Young recommends the use of but three media, discarding the dextrose broth.

The method of cross-inoculation as above outlined appears to have several advantages with western waters at least, and probably with all waters, in that positive presumptive tests in all three media either directly or on cross-inoculation make it unnecessary to apply confirmatory tests. Furthermore, the results are obtained in 48 hours in case all media respond positively, while if only a portion respond, the confirmatory test is already started by the streak culture on the lactose plate. Experience seems to show that this method gives more reliable results than the use of a single presumptive medium and that the inhibitive effect of the bile and the overgrowths in lactose broth are counterbalanced.

The table on page 139c shows the comparative results of 140 analyses of samples that showed fermentation in less than 50 per cent of the first fermentation tubes.

COMPARISON OF LACTOSE BILE, LACTOSE BROTH, AND DEXTROSE BILE FOR ISOLATING ORGANISMS OF THE B. COLI GROUP FROM WATER SAMPLES

Total number of samples showing fermentation.....140
Total number of samples confirming B. coli.....116

Positive Results for	No. of Samples.	Per cent.
B. coli with lactose bile	95	82
B. coli with lactose broth and not in lactose bile	20	17.2
B. coli with lactose broth and not in dextrose bile	6	5.1
B. coli with dextrose bile and not in lactose bile	14	12.1
B. coli with lactose bile and not in lactose broth or dextrose bile	2	1.7
B. coli with Water Survey method missed by lactose bile method	21	18.0

SAMPLES THAT DID NOT CONFIRM

	Lactose Bile.	Lactose Broth.	Dextrose Bile.
Number of samples	1.	17	6
Per cent	.73	12.4	4.35

POSITIVE FERMENTATION TUBES

	Lactose Bile Plus	Lactose Bile Minus	Lactose Broth Plus	Lactose Broth Minus	Dextrose Bile Plus	Dextrose Bile Minus
No. of samples	302	371	404	269	397	276
Per cent	44.5	55.5	60	40	59	41

The U. S. Treasury Department, on the recommendation of a special commission of experts appointed for the purpose by the Secretary of the Treasury, adopted on October 21, 1914, the following standard for drinking water to be supplied to the public by

common carriers in interstate commerce. (Public Health Reports, XXIX, 2959, Nov. 6, 1914.)

"The following are the maximum limits of permissible bacteriological impurity:

"1. The total number of bacteria developing on standard agar plates, incubated 24 hours at 37 degrees C., shall not exceed 100 per cubic centimeter, provided that the estimate shall be made from not less than two plates, showing such numbers and distribution of colonies as to indicate that the estimate is reliable and accurate.

"2. Not more than one out of five 10-c.c. portions of any sample examined shall show the presence of organisms of the bacillus coli group when tested as follows:

"(*a*) Five 10-c.c. portions of each sample tested shall be planted, each in a fermentation tube containing not less than 30 c.c. of lactose peptone broth. These shall be incubated 48 hours at 36 degrees C. and observed to note gas formation.

"(*b*) From each tube showing gas, more than 5 per cent of the closed arm of fermentation tube, plates shall be made after 48 hours' incubation, upon lactose litmus agar or Endo's medium.

"(*c*) When plate colonies resembling B. coli develop upon either of these plate media within 24 hours, a well-isolated characteristic colony shall be fished and transplanted into a lactose-broth fermentation tube, which shall be incubated at 37 degrees C. for 48 hours.

"For the purposes of enforcing any regulations which

may be based upon these recommendations, the following may be considered sufficient evidence of the presence of organisms of the bacillus coli group.

"Formation of gas in fermentation tube containing original sample of water (*a*).

"Development of acid-forming colonies on lactose litmus agar plates or bright red colonies on Endo's medium plates, when plates are prepared as directed above under (*b*).

"The formation of gas, occupying 10 per cent or more of closed arm of fermentation tube, in lactose peptone broth fermentation tube inoculated with colony fished from 24-hour lactose litmus agar or Endo medium plate.

"These steps are selected with reference to demonstrating the presence in the samples examined of aërobic lactose-fermenting organisms.

"3. It is recommended as a routine procedure, that in addition to five 10-c.c. portions, one 1-c.c. portion, and one 0.1-c.c. portion of each sample examined be planted in a lactose peptone broth fermentation tube, in order to demonstrate more fully the extent of pollution in grossly polluted samples.

"4. It is recommended that in the above-designated tests the culture media and methods used shall be in accordance with the specifications of the committee on standard methods of water analysis of the American Public Health Association, as set forth in 'Standard Methods of Water Analysis.' (A. P. H. A., 1912.)"

At present the choice of a practical method seems

to lie between the lactose bile test and this Treasury Department procedure. It is to be hoped that the Committee on Standard Methods of the Laboratory Section will pronounce definitely in favor of one or the other at the earliest possible moment.

CHAPTER VII

SIGNIFICANCE OF THE PRESENCE OF THE COLON GROUP IN WATER

Colon Bacilli in the Intestines of the Lower Animals. The Bacillus coli is by no means confined to the human intestine. Dyar and Keith (Dyar and Keith, 1893) found it to be the prevailing intestinal form in the cat, dog, hog, and cow. About the same time, Fremlin (Fremlin, 1893) found colon bacilli in the fæces of dogs, mice, and rabbits, but not in those of rats, guinea pigs, and pigeons. Smith (Smith, 1895) recorded the presence of the organism, in almost pure cultures, in the intestines of dogs, cats, swine, and cattle; and he also found it in the organs of fowls and turkeys after death. Brotzu (Brotzu, 1895) reported B. coli and allied forms as very abundant in the intestine of the dog; and Belitzer (Belitzer, 1899) isolated typical colon bacilli from the intestinal contents of horses, cattle, swine, and goats. Moore and Wright (Moore and Wright, 1900) recorded the finding of the colon bacillus in the horse, cow, dog, sheep, and hen; and in a later report (Moore and Wright, 1902) they noted its occurrence in swine and in some, but not all, the specimens of rabbits examined. In frogs it was not found. Eyre (1904) has more recently isolated typical B. coli from the intestines

of mice, rats, guinea pigs, rabbits, cats, dogs, sheep, goats, horses, cows, hens, ducks, pigeons, sparrows, divers, gulls, and fish of various sorts. Houston (1904) found B. coli abundant in the fæces of gulls, as might be expected from their feeding habits. Houston (1905) and other recent observers have found it impossible, even by the use of elaborate series of fermentation tests, to distinguish human B. coli from those found in animals. Savage (1906) compared colon-like organisms isolated from the intestines of swine, cattle, horses, and sheep with those of human origin in respect to their action upon lactose, dulcite, mannite, raffinose, glycerine, maltose, galactose, lævulose, saccharose, starch and cellulose; but he failed to find any general correlations between habitat and biochemical powers.

Ferreira, Horta and Paredes (1908[b]) have made perhaps the most elaborate study of the distribution of colon bacilli in the lower animals. They isolated 81 lactose-fermenting bacilli from 38 species of mammals and 8 species of birds, including monkeys, bears, wolves, foxes, hyenas, lions, panthers, tapirs, a camel, deer, and ostriches from the Zoological Gardens. These cultures were studied by an elaborate series of tests and 93 per cent of them proved to be typical B. coli. Bettencourt and Borges (1908[b]) working in the same laboratory showed that there were no specific differences in agglutination with immune sera and in complement fixation between the colon bacilli of human and of animal origin. Konrich (1910) reports the examination of 170 samples of fæces from men, horses, swine, sheep, cows, goats, dogs, cats, guinea pigs, mice, rabbits,

rats, earthworms, moles, fowls, swallows, sparrows, ducks, pigeons, geese, a jackdaw, a redstart, a blackbird, an adder, and a trout. Three out of 5 guinea pig samples, 4 out of 20 horse samples, 2 out of 3 mouse samples, 3 out of 8 rabbit samples, and 2 out of 8 earthworm samples, 14 in all, were negative; while all the rest showed B. coli.

In cold-blooded animals the occurrence of B. coli is less constant. Negative results in the frog and positive results in certain fishes, an adder and earthworms have just been quoted. Amyot (1902) failed to find the organism in the intestines of 23 fish representing 14 species. Johnson, on the other hand (Johnson, 1904), in the examination of the stomach and intestines of 67 fish caught in the polluted Illinois and Mississippi Rivers, isolated B. coli 47 times. He concluded from these results that the migration of fish from a contaminated stream or lake to an unpolluted one may explain the occasional finding of B. coli in small samples, or the more regular detection of it in large volumes of the water.

Bettencourt and Borges (1908[b]) isolated 29 cultures of colon-like microbes from the intestines of 17 types of fishes, reptiles and amphibia. Only 8 of the 29 formed gas in lactose broth and only 2 (from an eel and an adder) proved to be typical B. coli. It should be noted, however, that the samples of fæcal material were plated directly on Endo medium instead of being subjected to the more sensitive process of preliminary enrichment.

Fromme (1910) reviews the work of many observers

in regard to the presence of colon bacilli in the intestines of cold-blooded animals (particularly fish of various sorts and oysters) and concludes that while they are regularly found in warm-blooded animals they are found often, but not regularly, in cold-blooded animals. The lower the zoological type the rarer are the colon bacilli.

Alleged Ubiquity of the Colon Bacillus. Many bacteriologists have gone further and affirmed that the colon bacillus was not a form characteristic of the intestine at all, but a saprophyte having a wide distribution in nature. The first of this school, perhaps, was Kruse (Kruse, 1894), who in 1894 protested against the arbitrary conclusions drawn from the colon test as then applied. He pointed out that the characters usually observed marked, not a single species, but a large group of organisms. As ordinarily defined, he added, "the Bacterium coli is in no way characteristic of the fæces of men or animals. Such bacteria occur everywhere, in air, in earth, and in the water, from the most different sources." Even if the relations to milk and sugar media be considered, "micro-organisms with these characteristics are also widespread." Dr. Kruse gave no experimental data on which his opinion was based. In the same year Beckmann (Beckmann, 1894) isolated a bacillus which he identified by pretty thorough tests as B. coli from the city water of Strassburg, a ground-water which he believed could by no possibility be subject to fæcal contamination. Large quantities of water were used for the isolation.

Refik (Refik, 1896) recorded the constant presence

of colon bacilli in water of all sorts, public supplies, wells, cisterns, and springs in the neighborhood of Constantinople, and Poujol in the succeeding year reported (Poujol, 1897) the isolation of B. coli from 22 out of 34 waters studied by him in relation to their use as public supplies. The waters were from various sources—springs, wells, and rivers—but all were of fair quality and many quite free from any possibility of contamination. Samples of 100 c.c. were used for analysis.

Certain Italian observers appear to have come to even less conservative conclusions. Abba (Abba, 1895) found colon bacilli constantly present in unpolluted waters near Turin. Moroni (Moroni, 1898; Moroni, 1899) reported the examination of numerous deep and shallow wells and unpolluted springs about Parma, as well as of the public water-supply of the city, and concluded that the colon bacillus was a water form and had no sanitary significance. The characters used for the identification of the species in this case were fairly exhaustive, but both Abba and Moroni used liter samples for analysis.

Levy and Bruns (Levy and Bruns, 1899) gave a new turn to the discussion by emphasizing the importance of animal inoculation, already suggested by Blachstein (Blachstein, 1893) and others. They claimed that the existence of numerous para-colon and para-typhoid organisms in air, in dust, and in unpolluted water made it impossible to decide by ordinary bacteriological methods whether true colon bacilli were present in water or not. In no case, however, did representatives

of the colon group isolated by them from water kill a guinea pig, even when 1 or 2 c.c. were injected intraperitonally. The authors, therefore, considered pathogenicity as an attribute belonging only to the true B. coli of the intestine. This paper aroused Professor Kruse's pupil, Weissenfeld, to a publication, in which the position of the Bonn school was carried to an extreme. Weissenfeld reported (Weissenfeld, 1900) the analysis of 30 samples of water supposedly pure, and of 26 samples considered to be contaminated. In each case a single centimeter sample was first incubated in Parietti broth, and if no growth occurred, larger samples of half a liter or a liter were examined. Colon bacilli were found in all the samples; and the pathogenicity varied independently of the source of the water. The author concluded that " the so-called Bacterium coli may be found in waters from any source, good or bad, if only a sufficiently large quantity of the water be taken for analysis."

With regard to the question of pathogenicity as a diagnostic test for intestinal B. coli, there is little doubt of the correctness of Weissenfeld's conclusions. This property is so variable as to have no important value. Colon bacilli freshly isolated from the intestine are frequently non-virulent, and Savage (1903[a]) and others have shown that there is in general no correlation between pathogenic power and direct or indirect intestinal origin. On the other hand Weissenfeld's work entirely fails to show that the colon bacillus, pathogenic or non-pathogenic, is a normal inhabitant of unpolluted waters. Even his own results, if the quantitative rela-

tions be considered, furnish evidence to the contrary. In 24 of the 26 samples from bad sources, he isolated his imperfectly defined colon bacilli from 1 c.c. of the water, while in only 8 of the 30 samples of good waters could he find such organisms in that quantity.

Colon Bacilli on Plants and Plant Products. The work of certain recent observers has suggested the possibility that the colon bacillus may live in a semi-parasitic fashion on plants as well as on animals. Of a series of 47 cultures of lactic-acid bacteria, recently examined by one of ourselves (Prescott, 1902[a]; Prescott, 1903, Prescott, 1906), 25 were found to give the reactions of B. coli. These organisms were isolated chiefly from cereals and products of milling, such as flour, bran, cornmeal, oats, barley, etc., while others were in technical use for producing the lactic fermentation. There is no evidence that any of these organisms were of intestinal origin, and yet they possess all the characters of typical colon bacilli, even to the pathogenic action when inoculated into guinea pigs. In Germany, Papasotiriu (Papasotiriu, 1901) was meanwhile carrying on almost exactly similar investigations to Prescott's, with identical results.

Other testimony is somewhat conflicting with regard to the occurrence of B. coli on plants. Klein and Houston (1900) reported the finding of typical colon bacilli in only 3 out of 24 samples of wheat and oats obtained from a wholesale house; rice, flour, and oatmeal bought at two different retail shops gave B. coli in all three cereals in one case and on none in the other. Clark and Gage (1903) were unable to isolate B. coli

from standing grains. Gordan (1904) could not find B. coli in .1 and .01 mg. samples of clean bran, but isolated it easily from that of poor quality. Winslow and Walker (1907) have recently reported the examination of 178 samples of grain and 40 samples of grasses for B. coli without success. On the other hand, Düggeli (1904) found B. coli among the bacteria occurring on the leaves of growing plants, although it was not one of the most abundant species. Barthel, too (Barthel, 1906), found B. coli widely distributed on plants from both cultivated and uncultivated regions. Bettencourt and Borges (1908[a]) examined 35 samples of vegetables and cereals purchased in open market and found 12 lactose fermenters, of which 6 proved to be B. coli. It should be noted, however, that the method of isolation used was direct plating on Endo-medium, which is of course less sensitive than the enrichment processes used by other workers.

Neumann (1910) has recently studied the distribution of colon bacilli on and in various food substances such as bread, milk, butter and fruit. From fresh fruits immediately after picking he never isolated them, but they were present in a certain proportion of all the foods which had been exposed to human contamination and the author concludes that wherever human hands have been, there will B. coli be found. Konrich (1910) in a similar series of investigations obtained positive results from 46 out of 100 .1 to .5 gm. samples of cultivated plants while leaves of trees and grasses and herbs on waste places gave about 6 per cent positive results. Hay showed colon bacilli in 91 per cent of the 135 samples examined and grains in 55 per cent of 300 samples. Dr. L. A. Rogers of the U. S. Bureau of Animal Industry has recently obtained

results which indicate that the colon bacilli found on grains are of a distinct type, which can be distinguished from fæcal B. coli by their fermentative powers.

Colon Bacilli in Dust and Soil. Winslow and Kligler (1912) have shown that colon bacilli may be very abundant in the dust of city streets and houses, as might naturally be expected from the fact that such dust is largely made up of horse droppings. They examined 24 samples of street dust and 72 samples of house dust (all in New York City). All of the street dusts and 63 of the 72 house dusts contained colon bacilli in at least one of three duplicate $\frac{1}{100}$ gram portions. In two street samples the numbers rose to 330,000 and 660,000 per gram respectively, while the largest indoor result was 60,000. The average for the indoor dusts was between 1000 and 2000 per gram and for the street dusts over 50,000 per gram. This dust was dust deposited on surfaces and would only be carried up into the air by currents of some force. It is well known that colon bacilli are, as a matter of fact, rarely present in street or house air. Konrich (1910) exposed open Petri dishes of dextrose broth to the air of Jena streets for 24-hour periods, daily, for 3 months and found colon bacilli only 11 times. The colon bacilli in street dust may, however, perhaps account for the anomalous positive results sometimes obtained in reservoirs bordered by roadways.

Konrich (1910) has also made important contributions to the study of colon bacilli in the earth. Out of 547 samples of soil, 65% showed B. coli in portions of between .1 and .5 gm. The farther removed from cultivation a sample was, the less were the chances

of positive results. He concludes that B. coli is widely distributed in the outer world. It is almost always found in soil from cultivated fields or from traveled places. The farther a source is removed from travel and from cultivation the more rarely is the colon bacillus found; but it is never altogether absent. On plants or parts of plants it is frequently found when they come from cultivated land; on plants from waste places it is rarely found. It seems probable that colon bacilli may be even more widely distributed in a thickly settled and intensively cultivated country like Germany than in the United States.

The Number of Colon Bacilli, not Their Mere Presence, as an Index of Water Pollution. The more important practical conclusions to be drawn from these various investigations seem to be as follows:

1. Bacteria corresponding in every way to B. coli are by no means confined to animal intestines, but are widely distributed elsewhere in nature.

2. The finding of a few colon bacilli in large samples of water, or its occasional discovery in small samples, does not necessarily have any special significance.

3. The detection of B. coli in a large proportion of small samples (1 c.c. or less) examined is imperatively required as an indication of *recent* sewage pollution.

4. The *number* of colon bacilli in water rather than their *presence* should be used as a criterion of recent sewage pollution.

With these qualifications the value of the colon test was never more firmly established than it is to-day. Whether or not originally a domesticated form, it is

clear that the colon bacillus finds in the intestine of the higher vertebrates an environment better suited to its growth and multiplication than any other which occurs in nature. Houston (1903[a]) records the number of B. coli per gram of normal human fæces as between 100,000,000 and 1,000,000,000. It is almost certain that the only way in which large numbers of these organisms gain access to natural waters is by pollution with the domestic, industrial, and agricultural wastes of human life. If pollution has been recent, colon bacilli will be found in comparative abundance. If pollution has been *remote* the number of colon bacilli will be small, since there is good evidence that the majority of intestinal bacteria die out in water. If derived from cereals or the intestines of wild animals, the number will be insignificant except perhaps in the vicinity of great grain-fields or where the water receives refuse from grist-mills, tanneries, dairies, or lactic-acid factories.

The first recognition of the necessity for a quantitative estimation of colon bacilli in water we owe to Dr. Smith, who in 1892 (Smith, 1893[a]) outlined a plan for a study to be made by the New York Board of Health on the Mohawk and Hudson Rivers. Burri (Burri, 1895) pointed out that the use of so large a sample as a liter for examination would lead to the condemnation of many good waters. Freudenreich (Freudenreich, 1895) at the same time indicated the necessity for taking into account the *number* of colon bacilli present. He recorded the isolation of the organisms from unpolluted wells, when as large a quantity of water as 100 c.c. was used, and concluded that it was entirely absent only from

waters of great purity and present in large numbers only in cases of high pollution. This author also quoted Miquel as having found colon bacilli in almost every sample of drinking-water if only a sufficient portion were taken for analysis.

The practical results of the application of the colon test from this standpoint have proved of the highest value. As originally outlined by Dr. Smith, it consisted in the inoculation of a series of dextrose tubes with small portions of water, tenths or hundredths of the cubic centimeter. It was first used by Brown (Brown, 1893) in 1892 for the New York State Board of Health, and it showed from 22 to 92 fæcal bacteria per c.c. in the water of the Hudson River at the Albany intake, and from 3 to 49 at various points in the Mohawk River between Amsterdam and Schenectady. In some previous work at St. Louis, the colon bacilli in the Mississippi River were found to vary from 3 to 7 per c.c.

Hammerl (Hammerl, 1897) used the presence of Bacillus coli as a criterion of self-purification in the river Mur. He considered, in spite of the position taken by Kruse, that when a water contained large numbers of colon bacilli, as well as an excess of bacteria in general, it might be considered to be contaminated by human or animal excrement. As, however, the organism would naturally be present in large quantities of such a water as that of the Mur, he used no enrichment process, but made plate cultures direct; he defined the B. coli as a small bacillus, non-motile or but feebly motile, growing rapidly at 37° C., coagulating milk and forming gas in sugar media. In general, Hammerl

failed to find colon bacilli in the river by this method, except immediately below the various towns situated upon it; at these points of pollution he discovered a few colon colonies upon his plates, not more than 4 to 6 per c.c. of the water. He concluded that "the Bacterium coli, even when it is added to a stream in great numbers, under certain circumstances disappears very rapidly, so that it can no longer be detected in the examination of small portions of the water."

The most important work upon the distribution of B. coli has been that carried out in England by the bacteriologists of the local government board, by Dr. Houston in particular. This investigator (Houston, 1898; Houston, 1899[a]; Houston, 1900[a]) made an elaborate series of examinations of soils from various sources to see whether the microbes considered to be characteristic of sewage could gain access to water from surface washings free from human contamination. In the three papers published on this subject the examination of 46 soils was recorded. In only 10 of the samples was B. coli found, and of these 10, 9 were obviously polluted, being derived from sewage fields, freshly manured land, or the mud-banks of sewage-polluted rivers. The author finally concluded that "as a matter of actual observation the *relative abundance* of B. coli in pure and impure substances is so amazingly different as to lead us to suspect that not only does B. coli not flourish in nature under ordinary conditions, but that it tends to even lose its vitality and die." "In brief, I am strongly of opinion that the presence of B. coli in any number, whether in soil or in water, implies *recent* pollution of

animal sort." Pakes (Pakes, 1900) stated on the strength of an examination of "about 300 different samples of water," no particulars being published, that water from a deep well should not contain B. coli at all, but that water from other sources need not be condemned unless the organism was found in 20 c.c. or less. When colon bacilli were found only in greater quantities than 100 c.c. the water might be considered as probably safe. Horrocks (Horrocks, 1910), after a general review of English practice, concluded that "when a water-supply has been *recently* polluted with sewage, even in a dilution of one in one hundred thousand, it is quite easy to isolate the B. coli from 1 c.c. of the water." "I would say that a water which contained B. coli so sparingly that 200 c.c. required to be tested in order to find it had probably been polluted with sewage, but the contamination was not of recent date." Chick (Chick, 1900) found 6100 colon bacilli per c.c. in the Manchester ship canal, 55–190 in the polluted River Severn, and numbers up to 65,000 per gram in roadside mud. On the other hand, of 38 unpolluted streams and rivulets, 31 gave no Bacillus coli and the other 7 gave 1 per c.c. or less. The Liverpool tap water, snow, rain, and hail showed no colon bacilli.

One of the first elaborate applications of the colon test was made by Jordan in the examination of the fate of the Chicago sewage in the Desplaines and Illinois Rivers. In these studies of self-purification (Jordan, 1901) the analyses were made quantitative by the examination of numerous measured samples, fractions of the cubic centimeter; and the method employed

was enrichment, either in dextrose-broth fermentation tubes or in phenol broth, with subsequent plating on litmus lactose agar. The cultures isolated were tested as to their behavior in dextrose broth, peptone solution, milk, and gelatin; of the dextrose tubes made directly from the water all were considered positive which gave more than 20 per cent gas in the closed arm, with an appreciable excess of hydrogen. The results were very significant. In fresh sewage a positive result was obtained about one-third of the time in one one-hundred-thousandth of a cubic centimeter and almost constantly in one-ten-thousandth of a cubic centimeter. The Illinois and Michigan canal proved almost as bad, giving positive results on 7 days out of 28 in dilutions of one in a hundred thousand and on 28 days out of 32 in a dilution of one in ten thousand. At Morris, 27 miles below Lockport, where the canal enters the bed of the Desplaines River, and 9 miles below the entrance of the Kankakee, the principal diluting factor, the numbers were so reduced that positive results were obtained only on 11 days out of twenty in one-thousandth of a cubic centimeter, on 20 days out of thirty in one-hundredth of a cubic centimeter, and on 20 days out of 23 in one-tenth of a cubic centimeter. At Averyville, 159 miles below Chicago, colon bacilli were isolated on only 4 days out of 27 in one-tenth of a cubic centimeter, and on 13 days out of 31 in one cubic centimeter. A comparison with certain neighboring rivers showed this to be about the normal value for waters of similar character, as the following table extracted from Professor Jordan's paper will show:

NUMBER OF B. COLI PRESENT IN CERTAIN RIVER WATERS

(JORDAN, 1901)

Source of Sample.	0.1 c.c.		1 c.c.	
	No. Days Water Examined.	No. Days B. Coli Found.	No. Days Water Examined.	No. Days B. Coli Found.
Illinois River, Averyville...	27	4	31	13
Mississippi River, Grafton..	34	10	35	23
Fox River.....	22	2	23	6
Sangamon River.....	25	14	27	21
Missouri River.....	32	13	31	21

These results harmonize rather closely with those previously recorded by Brown and Fuller and indicate that in the larger rivers where the proportionate pollution is not extreme, colon bacilli may be isolated in about half the 1-c.c. samples examined. Such rivers are of course inadmissible as sources of water-supply, according to modern sanitary standards, unless subjected to purification of some sort.

Hunnewell and one of us (Winslow and Hunnewell, 1902[b]) examined a considerable series of normal waters for B. coli, testing 1 c.c. from each by the dextrose-broth method and a larger portion of 100 c.c. by incubation with phenol broth as described in Chapter VI. The samples were obtained from the public supplies of Taunton, Boston, Cambridge, Braintree, Brookline, Needham, and Lynn in Massachusetts, and Newport, R. I., from the Sudbury River, from the ocean, from the waters of springs bottled for the market, from ponds, pools of rain and melted snow, springs, brooks, shallow wells, and driven wells in various towns near

the city of Boston. For comparison 50 samples of polluted waters from the Charles, Mystic, Neponset, and North Rivers were examined. The colon bacillus was defined by gas production in dextrose broth, coagulation of milk, reduction of nitrates, formation of indol and failure to liquefy gelatin; and organisms which lacked the power to reduce nitrates or to form indol were classed in the "Paracolon group." The results are summarized in the following table:

PRESENCE OF B. COLI IN POLLUTED AND UNPOLLUTED WATERS

(WINSLOW AND HUNNEWELL, 1902[b])

Unpolluted Waters

	1 c.c.	100 c.c.
Samples examined	157	153
Dextrose broth positive	40	76
Lactose plate positive	13	31
Colon group	5	11
Paracolon group	5	5
B. cloacæ group	.....	5
Streptococcus group	3	10

Polluted Waters

	1 c.c.	100 c.c.
Samples examined	50	48
Dextrose broth positive	50	37
Lactose plate positive	50	26
Colon group	18	4
Paracolon group	6	....
B. cloacæ	1	....
Streptococcus group	25	22

As the authors pointed out, these tables indicate that bacteria capable of growth at the body temperature and fermenting dextrose and lactose are infrequently found

in unpolluted waters, and colon bacilli are very rarely present. In 157 samples, typical colon bacilli were found only 5 times out of 157, in 1 c.c. Lactose fermenting organisms appeared in only 8 per cent of the normal samples and in 100 per cent of the polluted ones, in 1 c.c. Incidentally it may be pointed out that these tables well illustrated the dangers of overgrowths, particularly in large samples. It is clear that the streptococci had killed out colon bacilli, originally present, in a large proportion of the 100-c.c. samples of polluted waters and in some of the 1-c.c. samples, since, in so many cases, gas formation was followed by the isolation of the streptococcus alone.

Colon Bacilli in Surface Waters. Clark and Gage (1903) have published the results of certain studies of

DISTRIBUTION OF TOTAL BACTERIA AND B. COLI IN SURFACE-WATERS

(Clark and Gage, 1903)

Lake.	Population of Watershed per Square Mile.	Bacteria per c.c.	B. Coli Per Cent Positive Tests.	
			1 c.c.	100 c.c.
1*	1400	612	13.3	33.0
2	356	319	3.5	17.2
3	116	103	0.0	0.0
4	90	170	0.0	14.0
5	62	87	0.0	9.0
6*	60	48	2.3	4.5
7*	50	66	4.6	21.0
8	47	133	0.0	9.0
9	42	131	0.0	6.7
10*	40	31	0.0	6.2
11	8	28	0.0	7.7
12	42	107	0.0	9.3

* Shores used for pleasure resorts.

Massachusetts ponds which indicate clearly the coincidence of the distribution of B. coli in single centimeters of surface waters, with actual sanitary conditions. They show also the slight significance of the test for this organism in larger volumes of water. Almost every source gave positive tests in 100 c.c., while with 1-c.c. samples only those lakes appear suspicious which are, in fact, exposed to dangerous pollution.

Houston (1905) gives the following table, which may be taken as another fair example of the distribution of B. coli in small streams and lakes. Of the two lakes studied, Loch Ericht is free from the pollution of human or domesticated animals, while Loch Laggan receives some drainage from farm lands; both are of large size. The brook and river samples were collected from adjacent streams.

DISTRIBUTION OF B. COLI IN SURFACE-WATERS

(HOUSTON, 1905)

Percentage of Samples showing B. Coli in each Dilution.

Dilution.	+0.1 c.c.	+1.0 c.c. −0.1 c.c.	+10 c.c. −1 c.c.	+100 c.c. −10 c.c.	Not in 100 c.c.
Brooks and river.	7.7	53.8	34.6	3.8	....
Loch Laggan....	.....	1.2	33.0	49.4	16.4
Loch Ericht.....	.....	.....	1.0	19.0	80.0

As an example of a heavily polluted stream, on the other hand, the table on page 159 may be cited. It shows in a striking way the increase of B. coli in the Thames on its passage through London and its progressive purification below.

The river at the lower stations in this table was considerably diluted with sea-water, yet it showed clearly its large proportion of sewage. Normal sea-water, even in the neighborhood of the shore, shows B. coli only in large samples. Houston (1904), in another communication, reports the examination of 168 samples of sea-water near the English coast. None of the samples showed B. coli in 1 c.c.; 97 samples gave negative results in 10 c.c.; 45 in 100 c.c., and 4 had no B. coli even in 1000 c.c.

B. COLI IN THE RIVER THAMES AT VARIOUS POINTS

(HOUSTON, 1904[a])

Percentage of Positive Results

Place.	−10 c.c.	+10 −1 c.c.	+1 −0.1 c.c.	+ .1 −0.01 c.c.	+ .01 −0.001 c.c.	+0.001 −0.0001 c.c.	+0.0001 −0.00001 c.c.
Sunbury.....	....	....	....	70.6	23.5	5.9	.....
Hampton.....	....	....	11.8	64.7	17.7	5.9	.....
Barking.....	....	....	....	4.2	45.8	45.8	4.2
Crossness.....	....	....	....	11.1	27.7	50.0	11.1
Purfleet.....	....	....	3.0	9.1	33.3	39.1	15.1
Grays.....	....	....	2.8	22.2	41.7	33.3	
Mucking.....	....	....	30.8	57.7	11.5	.....	
Chapman.....	5.0	45.0	50.0	....	....		
Barrow Deep ..	12.0	36.0	40.0	12.0	....		

Gärtner (1910) has collected some interesting data in regard to the ratio between the number of colon bacilli and of total bacteria in waters of different quality. The results from four different sets of experiments by Konrich at Jena, Houston at London, Noble in New York, and Hill at Giessen, may be combined as on the following page:

RATIO OF TOTAL BACTERIA TO COLON BACILLI IN WATERS OF DIFFERENT CLASSES

Coli titer—Smallest Portion of Water Showing B. Coli.	Ratio of Plate Count to B. Coli.					
	Jena.	London.		New York.		Giessen.
	20°	20°.	37°.	24°.	37°.	
100 c.c.	.....	60,500	11,800			
10 c.c.	695	15,100	1,810	1950	280	
1 c.c.	183	1,288	255	213	47	
0.1 c.c.	29.7	352	34	20	7	
0.01 c.c.	4.1	46	2.4	.....	.....	87
0.001 c.c.	.....	.....	.....	.....	.....	20
0.0001 c.c.	.....	.....	.....	.....	.....	4.9
0.00001 c.c.	.....	.....	.....	.....	.....	0.4

The rather regular decrease in the ratio of the total count to the B. coli count with an increase in the actual number of colon bacilli is very interesting.

Prof. Gärtner apparently holds that this fall in the ratio of the plate count to the "coli titer" indicates a fallacy in the method of the latter and in particular he emphasizes the absurdity of the lowest figures in the table which indicates that there were twice as many colon bacilli as bacteria of all sorts. It seems to us that the last phenomenon is quite as likely to be due to an error in the plate count as to a failure in the enrichment procedure. Unless dilutions are very carefully made plates inoculated with waters containing tens and hundreds of thousands of bacteria per c.c. are pretty likely to be so crowded that only a portion of the bacteria with which they are sown are able to develop. As to the diminishing ratio with increasing coli-content, it is exactly what might reasonably be expected. One-tenth to one-quarter of the bacteria in sewage may be

colon bacilli, and the greater the amount of sewage in water, the nearer this ratio will be approached.

Colon Bacilli in Ground-waters. With ground-waters the story is the same. Even in sources of excellent quality we should expect to find, and we do sometimes find, colon bacilli in large volumes of water. Abba, Orlandi, and Rondelli (1899) showed by experiments with B. prodigiosus at Turin that when bacteria are present in great numbers on the surface of the ground, a few may penetrate for a considerable distance and ultimately reach the sources of ground-waters. The chance that disease germs could survive this process in a soil so impervious as to allow colon bacilli to appear only in large samples of water, is infinitesimal.

An interesting contribution to the bacteriology of ground-waters was made by the Massachusetts State Board of Health (Massachusetts State Board of Health, 1901) in connection with the examination of the spring-waters bottled for the sale in the State. Ninety-nine springs were included in this study, and in almost every instance 4 samples were examined, 2 taken directly from the spring by the engineers of the board and 2 from the bottles as delivered for sale to the public. In the water of one spring B. coli was found twice, once in a sample from the spring and once in the bottled sample. This spring was situated in woodland, but was unprotected from surface drainage, and the method of filling bottles subjected it to possible contamination. In 5 other cases B. coli was found once in the sample from the spring; all were subject to pollution from dwellings or cultivated fields, and 4 of the 5 were shown

to be highly contaminated, chemically. In 7 other cases B. coli was found in the bottled samples alone; 3 of these sources were of high purity, but the bottling process furnished opportunity for contamination.

Clark and Gage (1903), in the examination of 170 samples of water from tubular and curb wells of good quality used as sources of water-supply, found B. coli only 5 times, once in 1 c.c. and 4 times in 100 c.c. Horton (1903), from a study of ground-waters in Ohio, concluded that the presence of B. coli in wells and springs was indicative of serious pollution.

Houston (1903[b]) makes an instructive comparison of some more or less polluted shallow wells at Chichester with deep ground-waters of high quality at Tunbridge Wells. The following table shows the value of the 1 cubic-centimeter sample in discriminating between good and bad waters.

DISTRIBUTION OF B. COLI IN GOOD AND BAD WELL WATERS

(HOUSTON, 1903[b])

Percentage of Positive Tests

Quantity of Water.	Chichester Shallow Wells.	Tunbridge Wells, Deep Wells.
100 c.c.	90	25
10 c.c.	80	6
1 c.c.	45	0
0.1 c.c.	20	0

In a subsequent investigation, Houston (1905) examined still larger samples of water from the Tunbridge Wells for B. coli: 49 samples of 100 c.c. each showed no B. coli, and 27 liter samples showed B. coli only once.

Kaiser (1905) reports an interesting correlation between total numbers and B. coli in a series of 38 well waters. Of 11 wells containing over 200 bacteria per c.c. 90 per cent showed colon-like organisms in liter samples. Of 12 wells containing from 50 to 200 bacteria per c.c. 67 per cent gave colon-like organisms; of 26 wells with less than 50 bacteria per c.c., only 27 per cent showed positive results.

Fromme (1910) brings out the relation between B. coli and total numbers in 120 samples of well waters near Hamburg in the table below.

RELATION BETWEEN TOTAL NUMBERS OF BACTERIA AND B. COLI

(FROMME, 1910)

Colony Count.	Number of Samples.	Per cent Positive B. coli Tests in 10 c.c.
Over 200	35	40.0
50–200	19	15.8
Under 50	66	3.0

Similar data obtained by one of us for some American sources have been cited in Chapter I. Even Konrich (1910), who is exceedingly skeptical as to the value of the colon test, has shown that an increase in the colon content of the Jena water supply (a ground-water) always followed a heavy rain which washed through some of the colon bacilli in the soil.

Colon Bacilli in Filtered Waters. One of the most important applications of the colon test is in the control of the operation of municipal water filters. It has been used for this purpose for 10 years or more at Lawrence, and Fuller laid stress upon its results in his classic experiments on water purification in the Ohio valley. At Cincinnati he records the presence of

colon bacilli in 60 per cent of the 1-c.c. samples from the Ohio River, while the effluent from either slow sand or mechanical filters gave positive results only half the time in samples of 50 c.c. The results of the examinations carried out at Lawrence for 6 years are brought together in the table below from the Annual Reports of the Massachusetts State Board of Health.

B. COLI IN MERRIMAC RIVER AND LAWRENCE FILTER EFFLUENT

	Merrimac River, Per cent of 1 c.c., Samples containing B. coli.	Merrimac River, Number B. coli per c.c.	Filtered Water, Per cent of 1 c.c., Sample containing B. coli.
1900	99.7	87	18.1
1901	*	*	*
1902	99.0	73	4.0
1903	99.0	78	4.2
1904	100.0	73	8.0
1905	100.0	118	4.7

* Not given.

At Harrisburg, Pa., mechanical filtration combined with chlorin disinfection has yielded the results tabulated below:

B. COLI IN RAW AND TREATED WATER AT HARRISBURG, PA.

(HARRISBURG, 1913)

Year.	Per Cent Positive Tests in 1 c.c.	
	Raw Water.	Treated Water.
1906	71.9	2.7
1907	64.0	1.0
1908	65.7	1.1
1909	63.1	1.0
1910	55.4	0.2
1911	77.3	0.6
1912	46.9	0.8

At Washington the most complete slow sand-filtration plant yet constructed has yielded the results tabulated below, for which we are indebted to the courtesy of Mr. F. F. Longley:

B. COLI IN POTOMAC RIVER AND WASHINGTON FILTER EFFLUENT

1906.	Dalecarlia Reservoir Inlet.			Filtered-Water Reservoir Outlet.		
	Number Tested.	Samples.		Number Tested.	Samples.	
		+ 10 c.c.	+ 1 c.c.		+ 10 c.c.	+ 1. c.c.
February....	15	5	3	24	0	0
March.....	24	12	3	27	0	0
April.....	18	9	6	25	1	0
May.....	25	3	1	27	0	0
June.....	26	9	8	26	0	0
July.....	20	8	9	21	1	0
August.....	26	21	14	27	1	1
September...	10	4	1	25	2	0
October.....	10	3	2	27	1	0
November...	8	3	0	25	2	0
December...	9	4	4	24	2	2
1907						
January.....	9	5	3	26	3	3
February....	8	2	2	23	0	0
March.....	8	7	4	26	0	0
April.....	9	4	1	26	1	0
May.....	23	21	15	26	0	0
June.....	25	20	17	25	0	0
July.....	26	11	8	26	0	0
August.....	27	13	8	27	0	0
September...	24	15	13	25	1	0

It must be remembered that in the Washington plant filtration is supplemented by thorough sedimentation, preliminary and subsequent. The entire credit for the good effluent obtained is not therefore due to the filters. At Lawrence it has been shown that removal of colon

bacilli in storage reservoirs and pipe systems may be considerable. The figures obtained in 1900 at various points in the distribution system may be cited as an example.

PERCENTAGE OF SAMPLES OF WATER CONTAINING B. COLI. LAWRENCE, MASS.

(Massachusetts State Board of Health, 1901)

	Effluent of Filter.	Outlet of Reservoir.	Tap City Hall.	Tap Experiment Station.
In 1 c.c.....	18	9	4	2
In 100 c.c. ...	38	23	16	16

Fromme (1910) in the examination of a filtered water averaging 35 bacteria per c.c. obtained the following results in various quantities of water.

PERCENTAGE OF SAMPLES OF WATER CONTAINING B. COLI.

(Fromme, 1910)

Number of Samples.	Volume Examined.	Per Cent Positive.
101	200 c.c.	30.9
412	10 c.c.	2.9
800	1 c.c.	0.25

In regard to the proportion of positive colon tests permissible in a filter effluent, Clark and Gage (Clark and Gage, 1900) reported some specially instructive observations made when certain of the underdrains of the Lawrence filter were relaid in the autumn of 1898. In doing this work the sand on some of the beds was seriously disturbed; and in December, after the work was completed, B. coli was found in 1 c.c. of the filtered

effluent in 72 per cent of the samples examined. In January and February the organisms were found in 54 per cent and 62 per cent of the samples, respectively, while in March the number fell to a normal value of 8 per cent. Corresponding to this excess of B. coli in the city water, there were 12 cases of typhoid fever in December, 59 cases in January, 12 in February, and 9 in March, all during the early part of the month. The authors conclude that "when filtering a river-water as polluted as that of the Merrimac, it is safe to assume that when B. coli is found only infrequently in 1 c.c. of the effluent, the typhoid germs, necessarily fewer in number and more easily removed by the filter, have been eliminated from the water."

The results of the daily tests carried out at municipal filter plants are frequently expressed in monthly or yearly averages, as in some of the cases quoted above. It must be remembered, however, that averages of this sort are accepted only by courtesy and with the implied assumption that conditions are approximately constant during the period averaged. When it is said that an acceptable effluent may show B. coli in 3 or 4 per cent of the samples tested the statement is true only for a series of samples collected and examined at the same time. If in a given month 3 per cent of the 1 c.c. samples tested show B. coli, the effluent may or may not be safe. If on each of 20 days 3 B. coli or thereabouts were present in 100 c.c. of the water it is probably a safe one. If on 19 days no B. coli were present, and on the twentieth day 100 c.c. showed 60 B. coli, the average result would be the same, but

the water on one day was of a dangerous character. With properly managed filter plants marked variations do not occur from day to day and average results are generally reliable. It is wholly misleading, however, to compare such results with the average examinations of an unfiltered surface water. With surface waters daily variations are the rule and a low monthly average of colon tests may include and cover up dangerous and significant high numbers at particular periods.

Summary of American and Foreign Opinion as to the Value of the Colon Test. The general results of the studies of the colon tests which have now been carried out in great numbers all over the world may be summarized by a few further citations.

In America the fact that the number of colon bacilli in a water measures the degree of its pollution is now universally accepted. The same conclusion has been established in England by the elaborate investigations of Houston and his pupils. Savage, for example, concluded (Savage, 1902) from a study of a large number of water supplies in Wales that even in surface waters, exposed to animal contamination from adjacent grazing grounds, B. coli is not present in 2 c.c. unless other pollution is present. In a more recent review of the whole subject, the same author (Savage, 1906) concludes that "there is no evidence or observations which have ever shown that B. coli, reasonably defined, is present in any numbers in sources which have not been exposed to some form of fæcal contamination."

In Germany, Petruschky and Pusch (Petruschky

and Pusch, 1903) examined a considerable series of waters from different sources by incubating measured samples with equal amounts of nutrient broth and isolating upon agar. In 45 samples of well-waters they found B. coli 7 times in .01 c.c., 9 times in .1 c.c., and 7 times in 1 c.c. In the other 22 cases it could not be found in 1 c.c. and in 4 cases not in 100 c.c. One sample showed it only in 600 c.c. and 1 not in 750 c.c. Of 29 river-waters, only 2 failed to give positive results in .1 c.c. and 14 showed B. coli in .001 of a c.c. or less. In sewage the number varied from 1 to 1,000,000 per c.c. The authors conclude that a quantitative estimation of the B. coli content furnishes a good measure of the fæcal pollution of water. There is still a school of bacteriologists in Germany, however, who are inclined to place little value on the colon-test. We have pointed out how Kruse and his pupils at Bonn led in the attack on it in 1894. Fourteen years later Kruse (1908) concluded after a full summary of the literature that the colon test was on the whole less valuable than the gelatin count, although he admitted that when the test is made quantitative it is valuable as a supplement to the plate count. Konrich (1910), working in Gärtner's laboratory at Jena, after perhaps the most exhaustive study ever made of the whole subject, concludes, that to include the colon test in forming judgment on the sanitary quality of a water is to complicate the procedure without improving it and that one would do well to omit the test except in certain special cases.

The German criticisms of the colon test are based

mainly on two considerations, the inaccuracy of the method itself and the difficulties in its interpretation. They hold on the one hand that the errors in the enrichment process and the consequent lack of correspondence between duplicate determinations are so great that the whole process is worthless. It is of course true that chance errors of distribution and the overgrowth which often occurs in the lower tubes of a dilution series do occasionally lead to individual erroneous results. If several dilutions are made with duplicates in each dilution, and particularly if reliance is placed, as it should be placed, not on single determinations, but on the average of several tests, results are, however, obtained which are in accord with each other and with the results of practical epidemiological experience.

The other objection brought forward by many German sanitarians is that the wide distribution of the colon bacillus leads to its presence even in considerable numbers in waters which are really of good sanitary quality. It is undoubtedly true that colon bacilli are often found in surface waters which receive no sewage but which are polluted only with the wash from roadways or cultivated land. Even dust blowing in from a roadway may perhaps contribute an appreciable pollution, as we have pointed out above. Gärtner (1910) calculates that in the soil of a cultivated field 100 meters square there are 15,000,000,000 colon bacilli and points out that it is no wonder that a rain should wash a few of them through into neighboring wells. Konrich (1910) goes so far as to say that to reject on principle all water containing B. coli in 1 c.c. portions

is impossible, since many regions have no other water available.

On the other hand, it may be urged that it is better to be on the safe side if possible. There is no doubt that in the United States there is no difficulty in securing water supplies, either from the ground or by storage or filtration, which only rarely contain colon bacilli in 1 c.c. samples. It may, perhaps, be that in the thickly settled and intensively cultivated parts of Germany this is not the case. Where it is possible to obtain such waters we believe it to be wise to do so. Ground waters to which the colon bacilli from cultivated soil penetrate and surface waters which still contain many colon bacilli from fields and roadways may be generally inocuous but there is always the chance that infectious material may find its way to the soil and may enter, along with the intrinsically harmless colon bacilli of manure. The two most recent German contributions to the subject are on the whole favorable to the colon test if wisely and intelligently applied. Fromme (1910), from Dunbar's laboratory at Hamburg, concludes from a survey of the literature and from his own studies that " the finding of colon bacilli in water is a valuable indicator of the quality of the water " and recommends it particularly for ground-water spring waters and filter effluents; and Prof. Gärtner, the head of the Hygiene laboratory at Jena, after a thorough discussion of previous work, comes to a much more conservative conclusion than his pupil, Konrich (Gärtner, 1910). While emphasizing the shortcomings of the process and deploring the tendency " to set B. coli on a high

throne and dance before it" he recognizes that the isolation of this organism has its place. "My aim will have been realized," he says, "if I have been able to show that the colon test may be useful under certain circumstances, but that it must be viewed with great caution and that, moreover, not the mere numbers of colon bacilli, but a careful consideration of the local situation and all the circumstances bearing on the special case are absolutely essential to the formation of an opinion." With Prof. Gärtner's emphasis on the importance of a sanitary inspection all experienced bacteriologists will certainly find themselves in agreement.

Some of the best French bacteriologists are strong supporters of the value of B. coli as an indicator of pollution. Gautié (1905) holds that the quantitative determination of B. coli is of the highest importance in water analysis; and Vincent (1905), in an excellent review of the subject, gives strong reasons for maintaining the same position. He finds B. coli absent from spring and well-waters of good quality and present in polluted water in proportion to its pollution. He concludes finally that water containing B. coli in .1 to 1.0 c.c. is unfit to drink, while if the organism is found in 1.0 to 10.0 c.c. it is of doubtful quality.

In Portugal too the trend of opinion is strongly in favor of the colon test—the Anglo-American procedure, as it is called in the publications of Dr. Bettencourt and his associates (Ferreira, Horta and Paredes, 1908[a]).

Altogether the evidence is quite conclusive that the absence of B. coli demonstrates the harmlessness of a

water as far as bacteriology can prove it. That when present, its numbers form a reasonably close index of the amount of pollution, the authors above quoted have proved beyond reasonable cavil. It may safely be said that when the colon bacillus is found in such abundance as to be isolated in a large proportion of cases from 1 c.c. of water, it is generally proof of the presence of serious pollution.

The standard here suggested is much more lenient than the one set forth by the U. S. Treasury Department for drinking water supplied to the public by common carriers (see p. 139*d*). Instead of permitting some 20 or 40 colon bacilli for each 100 c.c. of water as the ordinary test of five 1-c.c. samples does, the Treasury Department condemns a water containing over 2 colon bacilli in 100 c.c. It must be remembered, however, that this government standard was "recommended only for application to the special case in question"; and the Commission pointed out that, with water served on trains, it was necessary to set "narrower limits for permissible impurities of definitely dangerous character" than would be the case with waters where a sanitary inspection of the source could be made.

Mr. George W. Fuller and his colleagues, in testifying before the International Joint Commission on the Pollution of Boundary Waters, recommended a standard for raw waters before filtration of 500 B. coli per 100 c.c. of water expressed as an annual average and determined from a considerable number of confirmatory tests made throughout the year.

CHAPTER VIII

VARIETIES OF COLON BACILLI AND THEIR SPECIAL SIGNIFICANCE

Tests Used for Subdividing the Colon Group. The group of colon bacilli as defined in Chapter VI includes all aerobic Gram-negative bacilli which produce acid and gas in dextrose and lactose media. By the application of various tests, mainly bio-chemical, it may be further split up, almost at will, into a great number of varieties. The principal tests which have been used for this purpose are as follows:

1. Motility.
2. Coagulation of milk.
3. Production of indol.
4. Liquefaction of gelatin.
5. Reduction of nitrates.
6. Reduction of neutral red.
7. Fermentation of various carbohydrates other than dextrose and lactose.
8. Voges and Proskauer reaction.
9. Character of colonies on various solid media.
10. Aesculin reaction.
11. Growth and fermentation at 46°.

Motility is seldom determined in actual routine work in this country from the general belief that its demon-

stration is both burdensome and needless. Motility is a fluctuating and uncertain property and one which frequently requires repeated preliminary cultivations to make manifest. Furthermore, non-motile colon bacilli are common in the intestine and are probably as characteristic of pollution as the motile forms.

McWeeney (1904) found non-motile B. coli abundant in fæces and observed cases where the organisms were motile at 20° and not at 37°. He quotes Stöcklin as having found 116 non-motile strains among 300 otherwise normal B. coli from fæces. Evidence that non-motile bacteria, otherwise resembling B. coli, occur in unpolluted water would furnish the only basis for requiring this test as a routine procedure. No such evidence exists. The great body of data which connects the presence of B. coli with pollution includes all B. coli whether motile or not, since scarcely any bacteriologists observe this property in actual practice.

Howe (1912) has recently come to the conclusion that motility has no diagnostic value. MacConkey, however (MacConkey, 1909), after carefully reviewing the various characters suggested for use in studying colon bacilli, retains this one as important. He recommends that it be made in a drop of a 6-hour broth culture on an ordinary slide with a $\frac{1}{2}$-inch objective and dark ground illumination. Failure to show motility indicates in particular B. lactis-aerogenes and B. pneumoniæ of his classification (see p. 191).

Coagulation of milk is one of the most generally accepted tests for the colon group; and as a rule most lactose fermenting forms give a positive reaction. The

common practice in this country is to incubate litmus milk tubes for 48 hours at 37° and then heat to boiling. Tubes which have not coagulated spontaneously frequently do so on heating. Biffi (1906) has pointed out that milk to be used for this purpose should not be sterilized in the autoclave. Temperatures above 100° so alter the milk as to make its coagulation much slower. Konrich (1910) concluded from his experiments that the coagulation of milk by the colon bacillus is often not due to acid production, but to the secretion of a specific coagulating ferment, since he found that the addition of an amount of acid similar to that produced by the organism failed to coagulate.

The production of indol in a peptone solution is another test very generally used in this country and in England as diagnostic of "typical" B. coli. The usual procedure has been to incubate a tube of an aqueous solution containing 1 per cent peptone and .01 per cent sodium nitrite for four days at 37° and to test for indol by adding 1 c.c. of a .02 per cent solution of sodium or potassium nitrite and 1 c.c. of a 1 to 1 solution of sulphuric acid. Both the tube and the reagents should be cooled on ice before mixing, and the tube should be left in a cool place for an hour afterward to allow time for the characteristic rose-red color of nitroso-indol to develop.

Marshall (1907) and other German and English workers have shown that this nitrite and sulphuric acid test for indol often gives incorrect results and that the test modified by Böhme from Ehrlich is both more sensitive and more accurate. Two solutions are used; No. 1 is

made up of 4 parts of para-dimethyl-amido-benzaldehyde, 380 parts of absolute alcohol, and 80 parts of concentrated HCl; No. 2 is a saturated aqueous solution of potassium persulphate; 5 c.c. of 1 is added to 10 c.c. of a broth culture and then 5 c.c. of 2 is added and the whole shaken. A red color indicates indol. MacConkey (1909) who was at first inclined to discard indol as a routine test, believes that when made in this way it is of much value.

Howe (1912), from a statistical study of 630 strains of intestinal colon bacilli, concluded that indol, ammonia, and nitrite tests were but slightly correlated with general vigor and had but slight classificatory significance. It does not necessarily follow, however, that this is true of the forms which occur in stored waters. The so-called " atypical B. coli " are of course rare in fæces but they may occur in sufficient numbers to be important in waters which have been remotely polluted.

The liquefaction of gelatin is another test generally applied in any detailed study of the colon group. The longer the tubes are kept the higher will be the proportion of positive results, for there are many slowly liquefying forms grading by almost imperceptible degrees into the commoner non-liquefying type. The table cited from Gage and Phelps (1903) on p. 186 shows that of a series of 1908 cultures from various waters, sewages, and shellfish 8 per cent liquefied gelatin in 4 days, 10 per cent in 7 days, 13 per cent in 10 days, and 17 per cent in 14 days.

The reduction of nitrates to nitrites has been used in the United States as one of the five standard tests for

B. coli, but it has never gained wide acceptance in England or Germany. The usual practice has been to incubate for 4 days at 37° and to test for nitrites by adding a drop of each of the following solutions in succession:

A.	Sulphanilic acid	.5 gram
	Acetic acid (25% sol.)	150.0 c.c.
B.	Naphthylamine chloride	0.1 gram
	Distilled water	20.0 c.c.
	Acetic acid (25% sol.)	150.0 c.c.

A red or violet coloration indicates the presence of nitrites.

In making the nitrite test it is important to remember the possibility that appreciable amounts of nitrite may be present in the media—either derived from the air or from the use of impure peptone (Wherry, 1905). In the case of the nitrite reaction control tubes should always be tested from the same batch of media and only a distinct red color should be considered positive. The nitrite test is particularly subject to variations of unexplained origin. Of two duplicate tubes inoculated in the same way, one may show a strong reaction and the other none.

The reduction of neutral red has been extensively used in England and less in this country. It has been referred to in Chapter VI. as one of the tests suggested for use as a presumptive indicator of the colon group as a whole. MacConkey (1909) concludes that both the nitrate test and the neutral red test should be dropped from the procedure used in identifying colon bacilli, since so many organisms give these reactions that they have little significance. Houston, however, in the important investigations which will shortly be discussed, used the

production of a greenish fluorescence in neutral red broth as one of his tests of "typical" B. coli and apparently found it valuable.

Fermentation of various carbohydrate media has become the most common method of subdividing the bacilli of the colon group during the last few years, largely as a result of the work of MacConkey. Sugar broths for this test are generally put up in fermentation tubes of some sort so that the gas formation may be observed and perhaps measured, while acid production may be indicated by the addition of litmus or accurately determined by titration. The old-fashioned fermentation tube with a bulb and a stand has given way in most water laboratories to a plain bent tube of even bore and, more recently, to a still simpler device, a small vial inverted in an ordinary test-tube of sugar broth. The air in the top of the vial is driven out on sterilization and the presence or absence of gas can be easily determined, although it is not possible to measure its quantity with accuracy. If an ordinary bent tube is used the amount of gas in the closed arm may be conveniently measured by the Frost gasometer (Frost, 1901). If a measurement of the gas ratio is desired a few centimeters of strong sodium or potassium hydrate are added and mixed with the broth by cautiously tipping the tube; a second measurement determines the amount of gas absorbed (assumed to be CO_2).

It has been pointed out in Chapter VI that the gas ratio appears to be a reaction of slight importance as thus determined.

The list of fermentable substances used by various

observers in classifying colon bacilli is a long one. It was pointed out by Smith (1893) long ago that saccharose divides these organisms into two groups, and Winslow and Walker (1907) have found that strains which attack saccharose generally ferment raffinose also. MacConkey (1909) has made an exhaustive study of the fermentative reactions of the group and suggests an elaborate classification based on the use of a long series of carbohydrate media.

Howe (1912), from his recent statistical study of 630 strains of intestinal bacilli, concluded that fermentation tests in mannite, dulcite, and starch media are of little value in the classification of colon bacilli, since they are not closely correlated with other characters. It should be noted, however, that he worked only with fresh intestinal strains and it is possible that types characterized by definite reactions in these media may be rare in fæces but may develop so as to be important in stored waters.

Two noteworthy studies of the fermentative reactions of the colon group have recently been published by Kligler and by Rogers, Clark, and Davis, which will be discussed in detail on pages, 200–200*b*.

The Voges and Proskauer reaction has been extensively used by MacConkey and his followers in England and by Bergey and Deehan (1908) in this country. After the carbon dioxide in the fermentation tube has been absorbed by caustic soda, if the tube be allowed to stand, an eosin-like color gradually develops in the open arm, due to the presence of acetyl-methyl-carbinol. West (1909) points out that the test used by Rivàs,

the boiling of 1-4 c.c. of a 48-hour dextrose broth culture with 5 c.c. of a 10 per cent caustic soda solution, is a quick method of obtaining the Voges and Proskauer reaction. A yellow color is produced under these conditions by the sugar alone, and a pinkish eosin-like color when the acetyl-methyl-carbinol is present. The reaction is hastened by shaking or blowing into the tube to promote oxidation. West confirms the conclusion of MacConkey and Bergey that this reaction is characteristic of the B. lactis-aerogenes and B. cloacæ types (saccharose positive, dulcite negative organisms).

The characters of colonies on various solid media, such as Endo agar or Conradi-Drigalski agar appear to be of minor importance, usually depending on one of the simple fermentative reactions for their differential value. The aesculin test and the Eijkman test (fermentation at 46°) have been discussed in connection with their use as enrichment procedures in Chapter VI.

Biological Significance of Variations in the Colon Group. The general view among water bacteriologists has been that forms differing from the "typical" B. coli in one or more respects represented original intestinal types weakened by a prolonged sojourn in an unfavorable environment. As Whipple says (Whipple, 1903), "The type form of Bacillus coli is one which can be defined within reasonably narrow limits, but when the organism has been away from its natural habitat for varying periods of time, and has existed under abnormal conditions, its ability to react normally to the usual tests appears to be greatly im-

paired. Its power to reduce nitrates may be lost, or on the other hand, may be increased; its power to produce indol may be lost, or on the other hand, it may be increased; its power to coagulate milk, even, is sometimes reduced, although seldom entirely lost; its power to ferment carbohydrates may be altered so that the amount of gas obtained in a fermentation-tube, as well as its ratio of H to CO_2, is quite abnormal. But in spite of all these facts, the bacillus tested may have been originally a true Bacillus coli."

The results obtained by Peckham (1897) suggest that the indol reaction in particular is highly variable. By successive daily transfers in peptone broth she was able to increase the amount of indol produced by normal B. coli, and by a longer continuance of the same process to again weaken and abolish the power of forming it. Gas formation too was slackened in the cultures grown for too many transfers in the same medium. Horrocks (1903) found that B. coli kept in unsterilized well-waters and tap waters and in sterilized sewage and Thames water for 2 to 3 months showed only a feeble indol production and a delayed action on milk and neutral red. These modified forms are sometimes called "atypical B. coli," or "para-colon bacilli," and Vincent gives them the picturesque name, "microbic satellites of B. coli."

Such anomalies are most frequent with cultures freshly isolated from water, and they may often be avoided, as Fuller and Johnson (1899) have shown, by subjecting the organism to a process of preliminary cultivation. For this purpose the American Public

Health Association Committee recommends three successive cultivations in broth at 20 degrees, each of 24 hours' duration, inoculation from the last broth tube of a gelatin plate which is incubated for 48 hours at 20 degrees, inoculation of an agar streak from one colony on the plate and incubation of this streak for 48 hours at 20 degrees.

Often, however, the differences between types of the colon group indicate something much more fundamental than temporary weakening due to unfavorable environment. In particular sudden more or less permanent mutations may suddenly appear. Twort (1907) reports that by continued cultivation in sugar media he was able to develop fermentative power in certain members of the Gärtner group which lacked such powers before.

The work of Massini, Müller, Sauerbeck, Konrich and others (well summarized by Konrich, 1910) has also shown that mutations capable of fermenting sugars may suddenly arise from a parent strain lacking this power. Burri (1910) has contributed to the same problem and has found that the latent power to ferment a given sugar is released by growing the organism on that particular sugar, but that as Konrich and the others show only a certain proportion of the cells develop this power. The most important recent studies of bacterial mutation have been made by Penfold. In his latest communication (Penfold, 1912) he shows that many bacteria of the colon-typhoid group produce a mutant capable of fermenting lactose, that all strains of the typhoid bacillus produce dulcite and

iso-dulcite mutants, that many paratyphoid and Gärtner group bacilli produce raffinose mutants, and that other mutations also occur. The general phenomena are the same in each case. A strain which normally fails to ferment a given carbohydrate is grown upon a solid medium containing that carbohydrate. As the colonies develop there appear upon them raised papillæ of a different consistency from the rest of the colony and colored red if litmus be present. Transplants from the papillæ give pure cultures of a strain fermenting the carbohydrate in question and forming no papillæ. Transplants from the other portions of the colony give the original strain, non-fermenting, but capable of producing fermenting mutants as before. The identity of derivative strain in all other respects has been made clear by exhaustive cultural tests and serum reactions; and Penfold has shown that the whole process may be repeated, starting from an isolated single cell.

The work of MacConkey and Clemesha, which will be discussed shortly, is based on the assumption that a great number of minute subdivisions of the colon group, whether they have arisen by the gradual modifying effect of an unfavorable environment, or by mutations, or in some other way, are for practical purposes fairly permanent entities which they describe and name as definite species.

Distribution of Types of the Colon Group in Waters of Various Kinds. The sanitary importance of a study of these minor types within the colon group depends on the assumption that a certain set of characters is

generally associated with forms fresh from the intestine and may therefore be called typical. Such "typical" B. coli are understood as a rule to be motile, to clot milk, produce indol, reduce nitrate and neutral red and to fail to liquefy gelatin. It seems clear that forms having these characters predominate in the intestine itself while differing or "atypical" forms bear to them somewhat the relation implied in Vincent's term, satellites. Houston (1903[a]) examined in detail 101 cultures of coli-like microbes isolated from fæces and found that 72 per cent of the cultures were typical in all respects, while 11 per cent more differed only in being non-motile. The remaining 17 per cent were atypical, reacting abnormally to milk, indol, neutral red, litmus whey or Capaldi and Proskauer's medium. In a later investigation, Houston (1904) made a careful study of the distribution of the atypical forms in fæces, sewage, polluted water, and the filtered water-supplies of London. According to his ingenious system of nomenclature, " fl " indicates an organism which produces green fluorescence in neutral red broth; " ag," one which forms acid and gas in lactose media; " in," one which produces indol; and " ac " one which acidifies and clots litmus milk. The combination of all these properties gives " Flaginac," or typical B. coli; " aginac" is a form which fails to reduce neutral red; " flagac," one which fails to form indol, etc. " Flaginac " B. coli form the great majority of coli-like microbes in fæces, but Houston found that in filtered water they are outnumbered by atypical forms, of which he recognized thirty-five distinct types.

PERCENTAGE OF CULTURES PASSING VARIOUS TESTS IN THE ROUTINE EXAMINATION FOR B. COLI AT THE LAWRENCE EXPERIMENT STATION OF THE MASSACHUSETTS STATE BOARD OF HEALTH (GAGE AND PHELPS, 1903)

Source of Culture.	Per Cent of Preliminary Fermentations Passing Wurtz Agar Plate.	Agar Streak.					Number of Cultures Inoculated into Confirmatory Media.	Per Cent of Cultures.																Per Cent of Total Cultures Called B. Coli.
		Number of Cultures Made.	Per Cent of Cultures.					Fermentation Tube, Dextrose Broth.		Coagulate Milk.	Reduce Nitrates.	Produce Indol.	Gelatin Stab.											
			Characteristic.	No Growth.*	Dull-wrinkled.	Viscous.		Produce Gas.	Produce Scum.				Not Liquefied.		Liquefied.									
													Characteristic.	Wrinkled.	Time.				Form.					
															4 Days.	7 Days.	10 Days.	14 Days.	Funnel.	Cup.	Stratiform.			
B. coli	....	..	+	0	0	0	..	+	0	+	+	+	+	0	0	0	0	0	0	0	0			
Merrimac River	....	273	86	0	13	1	236	81	13	86	82	73	69	1	21	25	28	30	10	4	16			59
Filtered water	76	1485	83	9	4	4	1236	84	3	90	76	71	83	4	4	7	9	13	5	5	3			58
Sewage, etc	....	160	87	4	8	1	140	75	0	88	70	75	84	1	9	9	10	15	6	2	7			59
Shellfish	72	312	64	33	2	1	210	68	3	91	67	75	84	0	9	9	9	16	6	2	8			43
Spring-waters	78	53	74	0	4	23	39	56	0	67	67	59	72	0	15	18	28	28	20	0	8			41
Ice	73	79	72	16	8	0	557	67	5	88	42	88	63	7	25	25	26	30	5	4	21			48
Totals	75	2362	81	11	5	3	1908	80	4	88	74	71	80	3	8	10	13	17	6	5	6			58

*Including cultures which failed to grow on agar and streptococcus cultures, giving a very scanty, non-characteristic growth.

On the whole, much of the English evidence tends to the assumption that the atypical forms, or "paracolon bacilli," generally represent weakened strains from the intestinal B. coli stock. As Savage says, "we know that nearly all the coli-like organisms in fæces are quite typical B. coli, that in sewage a good many atypical varieties are present, and that in contaminated water and soil the proportion present is still larger."

The data tabulated on p. 186 from Gage and Phelps (1903) lead to a similar conclusion. About 60 per cent of the cultures isolated from polluted river water, filtered water, and sewage proved to be typical B. coli, while 41 and 43 per cent of those isolated from spring water and shellfish, respectively, and 48 per cent of those from ice belonged in this class.

Contradictory results, indicating a higher proportion of typical forms outside the body than within it, have been obtained by Konrich (1910) in the examination of 2387 different strains isolated in about equal proportions from fæces, earth, and water. Of the 2387 coli-like microbes studied, 308 were excluded by microscopic examination (showing abnormal morphology or positive Gram reaction) or by their liquefaction of gelatin. The other 2079 strains were tested in sugar media and peptone water with the results tabulated on p. 188.

We are inclined to attribute these results of Konrich's largely to the technique which he employed. It seems to be clearly stated in his paper that he obtained his fæcal cultures by direct plating on solid media, while his earth and water samples were treated to preliminary

BIOCHEMICAL REACTIONS OF 2079 ORGANISMS OF THE COLON GROUP

(KONRICH, 1910)

Medium.	Reaction.	Percentage of Positive Results.		
		Fæcal Strains.	Earth Strains.	Water Strains.
Dextrose broth.....	Gas production.....	100	100	100
Dextrose broth, 46°....	Gas production.....	59	82	82
Lactose broth.	Gas production.....	77	87	92
Lactose broth.....	Acid production....	97	100	100
Milk.....	Coagulation.....	65	84	80
Neutral red dextrose agar.....	Fluorescence.....	54	68	65
Peptone solution.....	Indol production....	38	57	57
Endo agar.....	Deep red colonies with greenish luster	60	77	76

enrichment in sugar broths. Such an enrichment would undoubtedly tend to increase the proportion of typical B. coli. Konrich's own experiments on the storage of pure cultures of colon bacilli in water showed a general, though not invariable, relative increase of atypical forms as the sojourn in water became long continued. Houston (1911) has recently pointed out the danger that the selective action of enrichment media may confuse the normal relations of the bacterial flora; and in order to obtain a more accurate idea of the relations involved he made an elaborate study of raw water, stored water, and stored and filtered water by direct plating, preceded by the use of various physical concentration methods. The results indicated in the table on p. 189 are of interest.

CHARACTERISTICS OF DEXTROSE FERMENTING BACTERIA FROM RAW, STORED, AND FILTERED WATERS

(HOUSTON, 1911)

Source.	No. of Strains.	Per Cent Positive Results. Gas Production.								Indol.	Voges & Proskauer Reaction.
		Lactose.	Saccharose.	Dulcite.	Salicin.	Raffinose.	Adonit.	Inosit.	Inulin.		
Raw river water.....	320	76	49	35	57	44	15	7	0.3	63	15
Stored water..	232	57	40	31	35	25	6	0.4	..	66	6
Stored and filtered water.	225	69	24	34	51	25	5	3	..	40	6

The strains having the combination of positive reactions in lactose and peptone solution made up 53 per cent of those isolated from the raw waters, 46 per cent of those from the stored waters, and 34 per cent of those from the stored and filtered waters.

MacConkey's Classification of the Colon Group. MacConkey is not satisfied with this general classification of colon bacilli into typical and atypical forms, but wishes to go much further, believing that even the so-called "typical B. coli" should rather be considered a complex including a considerable number of definite individual types. After a detailed study of the reactions of the lactose-fermenting bacteria of fæces he outlined a new classification based on fermentative reactions in the rarer sugars (MacConkey, 1905). Using saccharose and dulcite he first divided the lactose-fermenting organisms into four groups as indicated in the table on p. 190.

PRIMARY SUBDIVISIONS OF THE COLON GROUP

Saccharose.	Dulcite.	Type.
−	−	B. acidi-lactici
−	+	B. coli (or B. communis)
+	+	B. neapolitanus (or B. communior)
+	−	B. aerogenes

The fourth group, fermenting saccharose, but not dulcite, was further subdivided by MacConkey into the B. coscoroba type, which does not liquefy gelatin or give the Voges and Proskauer reaction, the B. lactis-aerogenes type, which does not liquefy gelatin, but does give the Voges and Proskauer reaction, and the B. cloacæ type, which liquefies gelatin and gives the Voges and Proskauer reaction. Records of the prevalence of the four principal groups in human and animal fæces and in milk are given in MacConkey's two papers (1905 and 1909) as well as their relative numbers in a suspension of fæces in water after various intervals of time. The results do not, however, appear to us to justify any important practical conclusions.

In his later paper MacConkey (1909) carried the sub-division of the colon group much further. He isolated 497 lactose-fermenting bacilli from the fæces of man and animals, from sewage, water, grains, etc. All were Gram-negative, fermented lactose, coagulated milk and reduced nitrate. They were subdivided by their action on gelatin, pepton and various fermentable substances and by their motility into over 100 types of which the more important have received

names. The principal types of this classification are indicated in the table below:

MACCONKEY'S CLASSIFICATION OF THE COLON GROUP

(MACCONKEY, 1909)

Group.	No.	Name.	Liquefaction of Gelatin.	Motility.	Indol Production.	Fermentation of					Voges and Proskauer Reaction.
						Saccharose.	Dulcite.	Adonit.	Inulin.	Inosit.	
I.	1	.....	−	+	+	−	−	+	−	−	−
	2	B. acidi-lactici.....	−	−	+	−	−	+	−	−	−
	3	B. levans.....	+	+	−	−	−	−	+	−	+
	4	B. Grünthal.....	−	+	+	−	−	−	−	−	−
	5	B. vesiculosus.....	−	−	+	−	−	−	−	−	−
II.	34	B. coli communis.....	−	+	+	−	+	−	−	−	−
	35	B. Schafferi.....	−	−	+	−	+	−	−	−	−
III.	65	B. oxytocus perniciosus.....	+	−	+	+	+	+	+	+	+
	68	B. pneumoniæ.....	−	−	−	+	+	+	−	+	−
	71	.....	−	+	+	+	+	−	−	−	−
	72	B. neapolitanus.....	−	−	+	+	+	−	−	−	−
	73	.....	+	+	−	+	+	−	−	−	+
IV.	103	B. lactis aerogenes...	−	−	−	+	−	+	−	+	+
	104	B. gasoformans.....	−	−	−	+	−	+	−	±	±
	107	B. coscoroba.....	−	−	+	+	−	−	−	−	−
	108	B. cloacæ.....	+	+	−	+	−	−	−	±	+

In this country the MacConkey classification was first adopted by Bergey and Deehan (1908). These workers used 8 diagnostic characters, motility, indol production, liquefaction of gelatin, the Voges-Proskauer reaction, and the fermentation of saccharose, dulcite, adonite and inulin. They tabulated 256 different combinations of these 8 characters and in the examina-

tion of 92 colon-like bacilli from 50 samples of milk, 8 of sewage and 1 of kefir they found 43 of the possible combinations.

Copeland and Hoover (1911) have recently urged the importance of these fermentative reactions in the rarer carbohydrates in the study of the colon group. They confirm the positive Voges and Proskauer reaction reported by other observers for B. lactis-aerogenes and B. cloacæ and point out that B. lactis-aerogenes is the only form in a considerable series studied which gives a brown coloration in aesculin media in one day. On the other hand they record a positive dulcite reaction for B. lactis-aerogenes and B. cloacæ which is highly confusing and makes it difficult to interpret their results. Both these names according to the usage of MacConkey, which has been accepted for the past five years, are applied to dulcite-negative saccharose-positive organisms.

Still another classification of the colon group is Jackson's modification of MacConkey's scheme in which MacConkey's four primary groups are symmetrically subdivided according to reactions in mannite and raffinose with motility, indol production, nitrate reduction, liquefaction of gelatin and coagulation of milk as secondary differential characters (Jackson, 1911). Under each of the four groups, B. communior (MacConkey's B. neapolitanus), B. communis (MacConkey's B. coli), B. aerogenes (MacConkey's Group IV), and B. acidilactici, he distinguishes four types, A (fermenting both mannite and raffinose, B (mannite+, raffinose−), C (mannite−, raffinose +), and D (fermenting neither

mannite nor raffinose); and he indicates reactions in other media by subscript letters. These types with their subtypes are fully discussed in the last report of the Committee on Standard Methods of Water Analysis (1912).

Clemesha's Investigation of Stored Waters in India. The most suggestive contribution to this subject which has been made in recent years is a book by Major W. W. Clemesha of the Indian Medical Service on The Bacteriology of Surface Waters in the Tropics (Clemesha, 1912[a]), in which a vigorous argument is made for the MacConkey classification in practical water work. Major Clemesha's researches show the prevalence of considerable numbers of all of MacConkey's primary types in human and bovine fæces as indicated in the table below, although the relative proportions found in England and in India do not correspond very closely. Clemesha's percentages are of special importance because they are based in each case on over 1000 colonies.

RELATIVE PROPORTION OF MacCONKEY'S GROUPS IN HUMAN FÆCES AND IN COW DUNG

Group.	Human Fæces.		Cow Dung.	
	MacConkey.	Clemesha.	MacConkey.	Clemesha.
1	34	53	17	40
2	38	17	25	9
3	15	7	48	16
4	12	22	12	35

Both in human fæces and in cow dung Clemesha finds the prevailing types to be B. coli, B. Grünthal,

and B. coscoroba, the three together usually making up 75 per cent all the lactose-fermenting organisms present. A very interesting point brought out in these investigations was the occurrence of "epidemics," of particular types which at certain periods become suddenly frequent, usually prevailing in human fæces, cow fæces and water supplies at the same time. (It should be noted for the benefit of anyone studying Clemesha's book that the tabular classification of the colon group at the end contains a serious misprint. B. lactis-aerogenes, B. gasoformans, B. coscoroba and B. cloacæ are there given as saccharose negative, whereas they should be saccharose positive.) The discussion in the text, however, appears to refer to the orthodox MacConkey types. Clemesha (1912[a]) made a number of experiments on the relative resistance of the various lactose-fermenting types by placing fæcal emulsions, with or without sand, in shallow dishes in the sunlight and at various intervals isolating 10 colonies of the predominant types and working out their fermentative reactions. In general the experiments showed B. coli to be the dominant form at the beginning. It quickly disappeared, however, and after a few hours B. lactis aerogenes, B. acidi-lactici, B. cloacæ and others appeared. At the end of the experiments, often on the second day, B. Grünthal or B. cloacæ were generally the only forms surviving. In a long series of examinations of Red Hills Lake Clemesha obtained 138 colonies of lactose-fermenting organisms during rainy periods and of these 59 belonged to MacConkey's Group I, 10 to Group II, 14 to Group III and 55 to

Group IV. Of 280 colonies isolated during dry periods, 37 belonged to Group I, 22 to Group III and 221 to Group IV. When the forces of self-purification had been at work, Group II (B. coli) entirely disappeared and Group IV (B. cloacæ and B. coscoroba) was predominant. B. Grünthal was the commonest of the Group I forms. B. cloacæ was especially prevalent in bottom samples.

A study of a number of rivers in Bengal gave the results tabulated below.

RELATIVE PREVALENCE OF CERTAIN LACTOSE-FERMENTING TYPES IN BENGAL RIVERS

MacConkey Group.	Types.	Dry Weather, Dec.–June.	Wet Weather, July–Nov.
I.	B. Grünthal and B. vesiculosus	41	23
II.	B. coli communis	3	13
IV.	B. lactis aerogenes	7	19
Do.	B. cloacæ	11	4

There are many irregularities in Dr. Clemesha's results. For example, B. aerogenes, as well as the other representatives of Group IV, was more abundant in Red Hills Lake during dry periods than at times of rain. On the whole, however, it does seem clear that his results justify a general classification of the lactose-fermenting organisms into three main groups according to resistance. B. coli communis and B. oxytocus perniciosus (representing MacConkey's Groups II and III, both fermenting dulcite) are sensitive organisms found in numbers only where pollution is fresh. B. lactis-aerogenes, representing the subgroup of MacConkey's

Group IV which ferments adonit, but does not form indol or liquefy gelatin, occupies a somewhat intermediate position, appearing in waters which have been fairly recently polluted and later disappearing again. Finally B. Grünthal and B. vesiculosus (MacConkey's Group I, negative in both saccharose and dulcite) and B. cloacæ and B. coscoroba (of MacConkey's Group IV, dulcite negative and saccharose positive), are highly resistant organisms which occur in relatively high proportions in stored waters. B. cloacæ is most abundant in bottom sediments and B. Grünthal and B. vesiculosus in sunned surface-waters.

The moral drawn by Major Clemesha is that for Indian conditions with waters stored in warm sunned lakes and large rivers, where sensitive fæcal bacteria have ample opportunity to die out and resistant fæcal bacteria have an ample opportunity to multiply, it is not proper to condemn water containing any members of the colon group without distinguishing between the more and the less resistant forms. For example, he quotes 239 examinations of which only 74 showed no B. coli according to the English standard, which closely corresponds to our own, while 165 showed what we should call positive results. Of the 165, however, 69 contained only the highly resistant B. Grünthal and 59 contained mixtures of other forms not belonging to MacConkey's Group II (saccharose negative, dulcite positive). Thus of the 239 samples 31 per cent would have been passed by Houston's standard, 53 per cent would have been condemned by Houston's standard, although containing only resistant forms which Clemesha

believes to be unimportant, and 16 per cent would be condemned by Clemesha as containing true B. communis.

Major Clemesha does not claim that these results necessarily indicate any change of procedure in dealing with the waters of temperate climates. Indeed, the experience of English and American bacteriologists offers pretty conclusive evidence that waters so stored as to be safe do not contain large numbers of lactose-fermenting organisms of any type. In other tropical countries and perhaps in warm summer weather, the Indian conditions may possibly be duplicated (as we know they are in the case of the forms fermenting dextrose but not lactose); and the experiments reported in this book deserve the careful consideration of water bacteriologists and sanitarians.

The results obtained by Houston (1911) in London unfortunately do not correspond at all with these Indian data. Houston studied in detail the reactions of about 800 strains of dextrose-fermenting bacteria from raw river-water, stored water, and stored and filtered water. Comparison of the relative prevalence of types from these three sources ought to furnish some confirmation of Major Clemesha's conclusions, even although the extreme conditions of warmth and sunlight are lacking. We find, however, on careful study of the figures that they do not. The Houston types corresponding to B. communis, B. Schafferi and B. neapolitanus (sensitive forms) are on the whole but little more prevalent in the raw than in the stored and filtered waters. On the other hand the types corresponding to B. Grünthal, B. vesiculosus, B. cos-

coroba and B. cloacæ (Clemesha's resistant types) are less abundant in the filtered and stored than in the raw water. Houston's lactose-fermenting forms classified in MacConkey's four great groups show the relations indicated in the table below, which are almost the reverse of what should be expected if the dulcite-fermenting forms (Groups II and III) were indicative of recent pollution.

DISTRIBUTION OF MacCONKEY'S GROUPS IN RAW, STORED, AND FILTERED WATER AT LONDON

Group.	Reactions.	Type.	Percentage in		
			Raw Water.	Stored Water.	Filtered Water.
I	Saccharose−Dulcite−	B. acidi-lactici. . .	34	39	37
II	Saccharose−Dulcite+	B. coli.	23	25	38
III	Saccharose+Dulcite+	B. neapolitanus. .	15	26	9
IV	Saccharose+Dulcite−	B. lactis-aerogenes	28	10	16

Statistical Classification of the Colon Group. From a biological standpoint, there is a twofold difficulty with such a classification as that of MacConkey and Jackson. In the first place it is enormously complex, or soon becomes so, as new investigators add new diagnostic tests. In the second place, it is entirely arbitrary in its choice of the order in which particular tests are to be used in splitting up the group. Closely related forms may be widely separated if they chance to differ in the one respect first chosen for dichotomic division.

The best basis for a classification following natural biological lines seems to us to be the statistical method first

suggested by Andrewes and Horder (1906) and Winslow and Winslow (1908) in the study of the cocci. The essential point about this method is that the characters of the organisms studied are not considered independently, but in relation to each other. The individual reactions are first studied quantitatively in a considerable series of allied strains, so that those types of reaction which are manifested by a large number of strains may be distinguished from the rarer intermediate variations. In the second place, the correlations between different characters are used as a basis for grouping the types on the assumption that a coincidence in several characters indicates a closer relationship than any single character alone.

The statistical method has been applied to the colon group in three recent studies which promise at last to lay a foundation for a sound knowledge of the relationships of the colon group.

Howe (1912) in his investigation dealt with 630 strains of fresh intestinal colon bacilli. He concluded from his exhaustive study that in bacilli of this type isolated directly from stools, the characters of motility, indol formation, ammonia production, nitrate reduction, fermentation of mannite, dulcite, and starch were not sufficiently correlated with each other or with other characters to be of classificatory value. Dextrose, lactose, saccharose and raffinose he found to constitute a natural metabolic gradient, in the order named, fermentation of any member of the series implying fermentation of those preceding it. Fifty-three per cent of his strains fermented all four sugars, 5 per cent all

but raffinose, 41 per cent attacked dextrose and lactose only, and 1 per cent dextrose alone.

Rogers, Clark and Davis (Journal of Infectious Diseases, 1914, XIV, 411) have made a most important contribution to our knowledge of the colon group. The most striking point is perhaps the fact that when the gas produced by these organisms in dextrose broth is collected under a vacuum and analyzed with proper precautions, two groups may be clearly distinguished by the ratio of hydrogen to carbon dioxide produced. One well-defined group including half the B. communior strains, all the B. communis strains, one-fifth of the B. aërogenes strains and four-fifths of the B. acidi-lactici strains studied gave a constant ratio of about one part of carbon dioxide to one part of hydrogen, while the rest gave a ratio of over 1.4 to 1.0 and usually 2 to 1 or more. The high-ratio group was usually saccharose-positive, dulcite-negative. The low-ratio group included strains of more widely differing reactions.

In a subsequent study, Rogers, Clark and Evans (Journal of Infectious Diseases, 1914, XV, 100) present a most significant comparison of the types of colon bacilli present in milk and in bovine fæces, respectively. Of 125 strains isolated from milk, about half belonged to the high-ratio group defined above (presumably corresponding to B. aërogenes), while in 150 cultures from bovine fæces this high-ratio group was found only once. The organisms of the low-ratio group in both milk and bovine fæces were divided rather sharply into a saccharose-positive dulcite-positive type (presumably B. communior) and a saccharose-negative generally dulcite-negative type (presumably B. acidi-lactici).

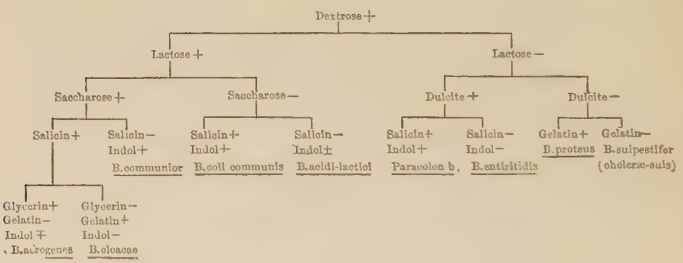
Kligler (Journal of Infectious Diseases, 1914, XV, 187) has analyzed the reactions of 80 strains of bacteria of the colon group in the collection of the American Museum of Natural History. His most important conclusion is, that after the primary division of the lactose-fermenting bacteria has been made on the basis of saccharose fermentation (separating B. communis and B. acidi-lactici on the one hand, from B. communior and B. aërogenes on the other), salicin rather than dulcite is the carbohydrate that is of next importance. The tables quoted below indicate that the salicin fermentation is much more closely correlated with the Voges and Proskauer reaction, the liquefaction of gelatin and the fermentation of glycerine than is that of dulcite.

CORRELATION OF DULCITE AND SALICIN FERMENTATION, RESPECTIVELY, WITH OTHER CHARACTERS OF BACILLI OF THE COLON GROUP. (KLIGLER, 1914.)

	Organisms.	Indol Positive.	V and P Positive	Glycerin Fermenters.	Gelatin Liquefiers.	Salicin Fermenters.
		%	%	%	%	%
Saccharose-positive Dulcite-positive	20	50	20	85	5	50
Saccharose-positive Dulcite-negative	20	25	70	70	15	90
Saccharose-negative Dulcite-positive	9	100	0	100	0	89
Saccharose-negative Dulcite-negative	8	63	0	88	0	38
						Dulcite Fermenters. %
Saccharose-positive Salicin-positive	28	21	68	68	14	35
Saccharose-positive Salicin-negative	12	75	0	100	0	83
Saccharose-negative Salicin-positive	11	100	0	100	0	73
Saccharose-negative Salicin-negative	6	50	0	83	0	17

Kligler, therefore, defines B. aërogenes as saccharose-positive salicin-positive (generally dulcite-negative); B. communior as saccharose-positive salicin-negative (generally dulcite-positive); B. communis as saccharose-negative salicin-positive (generally dulcite-positive); and B. acidi-lactici as saccharose-negative salicin-negative (generally dulcite-negative). B. cloacæ may be separated from B. aërogenes not only by the liquefaction of gelatin, but by the failure to ferment glycerine.

Kligler's general summary of the relationships of the dextrose fermenting bacteria is reproduced below:



Since Rogers and his colleagues did not use salicin in their tests, while Kligler did not determine the gas ratio, it is uncertain how far their results correspond, and it is to be hoped that their classifications may soon be compared in detail by the simultaneous application of both procedures. The fact that Rogers and his associates find the high-ratio group characteristic of milk but not of fæces, and their recent suggestion (at the Jacksonville meeting of the A. P. H. A.) that this high-ratio group is derived from grains and grasses may prove of the greatest significance in sanitary water analysis.

CHAPTER IX

OTHER INTESTINAL BACTERIA

It would be an obvious advantage if the evidence of sewage contamination, furnished by the presence of the colon group, could be reinforced and confirmed by the discovery in water of other forms equally characteristic of the intestinal canal. The attention of a few bacteriologists in England and America has been turned in this direction during the past few years; and two groups of organisms, the sewage streptococci and the anaerobic spore-bearing bacilli, have been described as probably significant.

Significance of the Sewage Streptococci. The term "sewage streptococci," as generally used, covers an ill-defined group, including many cocci which do not occur in well-marked chains. Those most commonly found grow feebly on the surface of ordinary nutrient agar, producing faint transparent, rounded colonies, but under semi-anaerobic conditions flourish better, giving a well-marked growth along the gelatin stab and only a small circumscribed film on the surface. They are favored by the presence of the sugars and ferment dextrose and lactose, with the formation of abundant acid but no gas. They are seen under the microscope as cocci, occurring as a rule in pairs,

short chains, or irregular groups. They do not show visible growth and do not form indol and nitrite in the standard peptone and nitrate solutions; most of them do not liquefy gelatin, though occasionally forms are found which possess this power. Until recently no systematic study of the various species found in the intestine had been made and all cocci giving the characteristic growth on agar and strongly fermenting lactose are commonly included as "sewage streptococci."

Although the significance of the streptococci as sewage organisms is not established with the same definiteness which marks our knowledge of the colon group, these forms have been isolated so frequently from polluted sources and so rarely from normal ones that it now seems reasonable to regard their presence as indicative of pollution. Although originally reported by Laws and Andrewes (Laws and Andrewes, 1894), their importance was not emphasized until 1899 and 1900, when Houston (Houston, 1899[b], 1900[b]) laid special stress upon the fact that streptococci and staphylococci seem to be characteristic of sewage and animal waste, the former being, in his opinion, the more truly indicative of dangerous pollution, since they are "readily demonstrable in waters recently polluted and seemingly altogether absent from waters above suspicion of contamination." In six rivers recently extensively sewage-polluted, he found streptococci in from one-tenth to one ten-thousandth of a c.c. of the water examined, although in some cases the chemical analysis would not have indicated dangerous pollution. On the other hand, eight rivers, not extensively polluted, showed

no streptococci in one-tenth of a c.c., although the chemical and the ordinary bacteriological tests gave results which would condemn the waters. Horrocks (Horrocks, 1901) found these organisms in great abundance in sewage and in waters which were known to be sewage-polluted, but which contained no traces of Bacillus coli. He found by experiment that B. coli gradually disappeared from specimens of sewage kept in the dark at the temperature of an outside veranda, while the commonest forms which persisted were varieties of streptococci and staphylococci.

In America attention was first called to these organisms by Hunnewell and one of us (Winslow and Hunnewell, 1902[a]), and the same authors later (Winslow and Hunnewell, 1902[b]) recorded the isolation of streptococci from 25 out of 50 samples of polluted waters. Gage (Gage, 1902), from the Lawrence Experiment Station, has reported the organisms present in the sewage of that city, while Prescott (1902[b]) has shown that they are abundant in fæcal matter and often overgrow B. coli in a few hours when inoculations are made from such material into dextrose broth. In the monograph of Le Gros (Le Gros, 1902) of the many streptococci described, all without exception were isolated, either from the body or from sewage. Baker and one of us (Prescott and Baker, 1904), found these organisms present in each of 50 samples of polluted waters. On the other hand, in the study of 259 samples of presumably unpolluted waters, by the method of direct plating, Nibecker and of the authors (Winslow and Nibecker, 1903) found streptococci in only one sample. Clemesha

(1912[a]) finds that streptococci in India are present in .0001 or .00001 gm. of fæces, but are rare in waters unless very grossly polluted. In a series of bottle experiments in the laboratory and in the study of an artificially polluted tank outdoors he showed that they disappear very rapidly in water, within 2 or 3 days at the most. Gordon (1904) showed that certain streptococci are abundant in normal saliva and are found in air which has been exposed to human pollution but not in normal air. On the whole there can be no doubt of the fact that streptococci occur on the surfaces of the human and animal body more commonly than anywhere else in nature.

Isolation of Sewage Streptococci. The isolation of these organisms either from plates or liquid cultures is easy. On the lactose-agar plate, made directly from a polluted water, the colonies of the streptococci may generally be distinguished from those of other acid-formers by their small size, compact structure, and deep-red color, which is permanent, never changing to blue at a later period of incubation. Developing somewhat slowly, however, they may be overlooked if present only in small numbers. In the dextrose-broth tube, streptococci will generally appear in abundance after a suitable period of incubation. Prescott and Baker, in the work above mentioned, found that with mixtures of B. coli and streptococci in which the initial ratios of the latter to the former varied from 1 : 94 to 208 : 1, the colon bacilli developed rapidly during the early part of the experiment, reaching a maximum after about 14 hours, and then diminishing

rapidly. The streptococci first became apparent after 10 to 15 hours and reached their maximum after 20 to 60 hours, according to the number originally present.

Applying the same method to polluted waters, similar periodic changes were observed; nearly pure cultures of B. coli were first obtained, then the gradual displacement of one form by the other took place, and at length

RELATIVE GROWTH OF B. COLI AND SEWAGE STREPTOCOCCI FROM POLLUTED WATERS IN DEXTROSE BROTH

(Prescott and Baker, 1904)

Sample Number			1	2	3	4	5	6	7	8	9	10
Red colonies developing from 1 c.c. of original sample on litmus lactose agar			4	10	9	5	8	55	35	460	1250	105
Number found, in millions per cubic centimeter, after growth in dextrose broth for various periods	11 hrs.	B. coli	0	20	68	200	185	400	130	332	420	410
		Strept	0	0	0	0	0	0	0	0	0	0
	16 hrs.	B. coli	200	76	130	270	220	210	140	420	285	410
		Strept	40	25	20	10	45	30	20	210	75	145
	23 hrs.	B. coli	280	150	385	370	300	570	200	405	320	300
		Strept	140	85	280	170	300	1700	110	350	370	350
	39 hrs.	B. coli	0	0	25	110	0	210	20	24	105	
		Strept	474	420	480	300	390	170	400	105	250	
	63 hrs.	B. coli	0	0	0	0	0	12	8	0	0	0
		Strept	2	0	0	45	1	2	45	150	86	170
First gas noted after (hrs.)			10	10	9	9	10	8	10	6	6	8

the streptococci were present either in pure culture or in great predominance as shown by the accompanying tables. The samples of water were plated directly upon litmus lactose agar and the plates were incubated at 37° for 24 hours, when the red colonies were counted. At the time of plating, 1 c.c. from each sample was also inoculated into dextrose broth in fermentation tubes.

which were likewise incubated at 37°. After various periods, as indicated by the tables below, the tubes were shaken thoroughly and 1 c.c. of the contents withdrawn. This was diluted (generally 1–1,000,000,) with sterile water, plated on litmus lactose agar in the usual way, and incubated for 24 hours. The colonies of B. coli and streptococci were distinguished micro-

RELATIVE GROWTH OF B. COLI AND SEWAGE STREPTOCOCCI FROM POLLUTED WATERS IN DEXTROSE BROTH

(PRESCOTT AND BAKER, 1904)

Sample Number			18	19	20	21	22	23	24	25
Red colonies developing from 1 c.c. of original sample on litmus lactose agar			1	150	25	30	50	170	200	30
Number found, in millions per cubic centimeter, after growth in dextrose broth for various periods	7 hrs.	B. coli	.02	....	....	.01	.04	.12	.55	1.6
		Strept.	0	....	....	0	0	0	0	0
	17 hrs.	B. coli	266	100	88	350	510	380	330	160
		Strept.	150	0	40	140	240	128	80	220
	27 hrs.	B. coli	520	610	72	700	1000	740	100	300
		Strept.	800	860	670	1080	2500	4380	....	3900
	40 hrs.	B. coli	0	0	10	22	36	7	7	
		Strept.	252	330	260	22	66	60	52	
	52 hrs.	B. coli	10	16	38	20	70	35	10	27
		Strept.	40	16	3.8	31	41	25	10	30

scopically, and by difference in color and general characters.

The successive growth of these two intestinal groups in the same dextrose-broth tube suggests the following method for the detection of both B. coli and sewage streptococci.

Inoculate the desired quantity of water, preferably

1 c.c., into dextrose broth, in a fermentation tube, and incubate at 37°. After a few hours' incubation examine the cultures for gas. Within 2 or 3 hours' after gas formation is first evident, plate from the broth in litmus lactose agar, incubating for 12 to 18 hours at 37°. If at the end of this time no acid-producing colonies are found, it is probably safe to assume that there were no colon bacilli present. On the other hand, if red colonies are developed, these must be further examined by the regular diagnostic tests for B. coli. After the first plating from the dextrose broth, replace the fermentation tube in the incubator and allow it to remain for 24 to 36 hours, then plate again on litmus lactose agar. This plating should give a nearly pure culture of streptococci if these organisms were originally present in the water.

Streptococci as Indicators of Recent Pollution. The comparative relation of the streptococci and the colon bacilli to sewage pollution is still somewhat uncertain. Houston (Houston, 1900) held that the former microbes imply "animal pollution of extremely recent and therefore specially dangerous kind," and Clemesha's experiments led to the same conclusion. Horrocks (Horrocks, 1901), on the other hand, maintains, largely on the strength of certain experiments with stored sewage, that the streptococci persist after colon bacilli have disappeared and indicate contamination with old sewage which is not necessarily dangerous. These discordant results are probably to be explained by the different media in which the viability of the bacteria was compared. It seems likely that in sewage where there is a

large amount of organic food material present the streptococci may kill out the colon bacilli as they do in the fermentation tube, and as we know they frequently do in shellfish. This would explain Horrocks' results. On the other hand, there is good evidence that the streptococci are less resistant than B. coli to the unfavorable conditions which exist in water of ordinary organic purity. In waters of potable character B. coli is frequently present without the streptococcus; and a negative test for streptococci has little significance. A positive test, on the other hand, furnishes valuable confirmatory evidence of pollution. This evidence is of course of special importance when through the activity of the streptococci themselves, or from any other cause the colon isolation has yielded an erroneous negative result.

The English Committee appointed to consider the standardization of methods for the bacterioscopic examination of water (1904) by a majority vote recommended the enumeration of streptococci, as a routine procedure in sanitary water analysis, but in this country the Committee on Standard Methods of Water Analysis (1912) has concluded that "the information afforded by the occurrence of these organisms seems to be of less value than in the case of B. coli and it is believed that for the present at least, the streptococcus test is of subordinate importance."

Use of the Streptococci to Distinguish between Human and Animal Pollution. There seems some reason to hope that the streptococci may prove of assistance in the important task of differentiating human and animal

pollution, a task in which all other tests have so far failed. Unlike the colon bacilli, streptococci from the intestines of cattle and men appear to belong to distinct types. The recognition of this fact we owe primarily to Gordon (1905), who made an elaborate study of the fermentative power of the streptococci in a long series of carbohydrate media. His work and that of Houston (Houston, 1904; Houston, 1905[a], Houston, 1905[b]) have made it clear that the streptococci of the herbivora differ from those found in the human body in their low fermentative power. In their review of the genus, Andrewes and Horder (1906) describe the type characteristic of the herbivora under the name, Str. equinus, and define it by its failure to ferment lactose, raffinose, inulin or mannite, or to reduce neutral red. Five other types are described from the human mouth and intestine; all of them ferment lactose, and most reduce neutral red and ferment raffinose. The commonest intestinal form clots milk, reduces neutral red and ferments saccharose, salicin, coniferin and mannite. The specific types of the genus Streptococcus, grade into each other by almost imperceptible degrees, and streptococci fermenting lactose and raffinose and reducing neutral red are sometimes found in bovine fæces; but the studies made in this country by Winslow and Palmer (1910) confirm the conclusions of the English observers that there are specific differences between the streptococci of the human, bovine, and equine intestines. The most important of these results are indicated in the table below:

COMPARATIVE FERMENTATIVE POWER OF STREPTOCOCCI FROM THE HORSE, THE COW, AND MAN

(Winslow and Palmer, 1910)

Streptococci.	Percentage of Positive Results (300 Strains).		
	Lactose.	Raffinose.	Mannite.
Human	62	6	28
Equine	8	4	2
Bovine	52	28	6

The rarity of lactose-fermenting streptococci in the horse makes it probable that this group can be used for distinguishing pollution by street washings from that due to domestic sewage; and the fact that a considerably larger proportion of human strains attack mannite and a considerably larger proportion of bovine strains ferment raffinose should make it possible to use the ratio between results in these two media to distinguish between the wash from pastures and cultivated land and sewage. Clemesha (1912[a]) in India found that both human and bovine fæcal streptococci fermented raffinose, saccharose, and salicin, but not mannite. Fuller and Armstrong, however (Journal of Infectious Diseases, 1913, XIII, 442), confirm the results obtained by Winslow and Palmer, finding that lactose-fermenting streptococci of any kind are rare in horse dung, that the streptococci of bovine fæces characteristically ferment lactose and raffinose, while the fermentation of lactose and mannite is usual among human fæcal strains. The results of these workers were more consistent than those tabulated above, 65 per cent of the human strains being of the S. fæcalis type (man-

nite-positive), while 64 per cent of the bovine strains were of the S. salivarius (raffinose-positive) type.

Rogers and Dahlberg (Journal of Agricultural Research, 1914, I, 491) also find that streptococci from bovine fæces typically ferment raffinose and not mannite, while those from the mouth of the cow are frequently of the S. fæcalis type.

The Anaerobic Spore-forming Bacilli. The English bacteriologists have ascribed much importance as indicators of sewage pollution to another group of organisms, the anaerobic spore-forming bacilli, of which the form described as B. aerogenes capsulatus (Welch and Nuttall, 1892) and now called B. welchii, and the form isolated by Klein (Klein, 1898; Klein, 1899) in 1895 in the course of an epidemic of diarrhœa at St. Bartholomew's Hospital, described under the name of B. enteritidis sporogenes (now called B. sporogenes) are types.

The procedure originally described by Klein for isolating B. sporogenes is as follows: a portion of the sample to be examined is added to a tube of sterile milk, which is then heated to 80° C. for 10 minutes to destroy vegetative cells. The milk is next cooled and incubated under anaerobic conditions, which may be accomplished most conveniently by Wright's method. A tight plug of cotton is forced a quarter way down the test-tube, the space above is loosely filled with pyrogallic acid, a few drops of a strong solution of caustic potash are added, and the tube is tightly closed with a rubber stopper. After 18 to 36 hours at 37° the appearance of the tube will be characteristic if the B. sporogenes

is present. "The cream is torn or altogether dissociated by the development of gas, so that the surface of the medium is covered with stringy, pinkish-white masses of coagulated casein, enclosing a number of gas-bubbles. The main portion of the tube formerly occupied by the milk now contains a colorless, thin, watery whey, with a few casein lumps adhering here and there to the sides of the tube. When the tube is opened, the whey has a smell of butyric acid and is acid in reaction. Under the microscope the whey is found to contain numerous rods, some motile, others motionless."

Since this organism is not present in very large numbers, even in sewage, the test of a water-supply must be made with large samples, and the concentration of at least 2000 c.c. of water by filtration through a Pasteur filter is recommended by Horrocks as a necessary prelude (Horrocks, 1901). The Committee on Standard Methods of Water Analysis (1912) recommends the following enrichment procedure for the isolation of B. sporogenes which avoids physical concentration. Various dilutions of the water to be tested are incubated in fermentation tubes containing liver broth for 24 hours at 37°. If B. sporogenes is present gas will be evolved and a characteristic "vile odor" will be produced. If this reaction is obtained the contents of each positive tube is transferred to an Erlenmeyer flask or large test-tube and heated at 80° C. for 10 minutes to destroy vegetative cells. One c.c. of broth containing sediment is withdrawn from the bottom of each flask and enriched once more in a fresh liver broth tube. B. sporogenes will now usually be present in pure culture showing

large sluggishly motile bacilli containing spores. A gelatin stab culture made from these 24-hour liver broth tubes will show after 48 hours incubation at 20° a distinct liquefying anaerobic growth beginning about 2 cm. below the surface with gas bubbles at the top of the liquefied area. In order to obtain absolutely pure cultures it is necessary to fish from liver broth tubes only 3–5 hours old as only young vegetative cells will grow on plates. Transplants from the closed arm of such tubes will grow on dextrose liver agar plates incubated under anaërobic conditions.

Another method for isolating B. sporogenes suggested by Wells is simpler than the foregoing. He recommends the use of freshly-boiled lactose broth tubes (heated in Arnold Sterilizer) brought to a temperature of 70 degrees C., at which temperature the inoculation is made. The inoculated tubes are then kept at 70 degrees C. for 10 minutes and subsequently incubated at 37 degrees C. By this means the spore forms of B. sporogenes develop into vegetative cells in the course of 24 hours and give rise to a rapid evolution of gas. Aërobic plates made from these tubes will generally give no growth. On the other hand, if B. coli were present, gas would ordinarily be produced in 18 hours and aërobic plates, either on litmus agar or Endo medium would show an abundance of colonies.

The organisms of the B. sporogenes group are large stout bacilli often occurring in chains. They liquefy gelatin vigorously and on agar produce fine discrete gray colonies. They vigorously ferment dextrose, lactose and saccharose, producing acid and gas, and in sugar

agar each colony will be marked by one or more gas bubbles surrounded by a delicate whitish fringe. The organism is strongly pathogenic for guinea pigs, by which character it is distinguished from the B. butyricus of Botkin. B. welchii differs from B. sporogenes chiefly in lacking motility and in forming spores with less readiness (Klotz and Holman, 1911).

The researches of Klein and Houston (Klein and Houston, 1898, 1899) have shown that the B. sporogenes occurs in English sewage in numbers varying from 30 to 2200 per c.c. and that it is often absent in considerable volumes of pure water. In Boston sewage it may usually be isolated from .01 or .001 of a c.c. (Winslow and Belcher, 1904). Since the spores of an anaerobic bacillus may persist for an indefinite period in polluted waters, their presence need not necessarily indicate recent or dangerous pollution.

Vincent (1907) and other French observers consider the determination of the total number of anaerobic bacteria as significant, since the decomposition of organic matter is accompanied by anaerobic growth. It is not claimed, however, that bacteria of this type are characteristic of animal more than of vegetable decompositions, and the total anaerobic count apparently adds nothing of importance to the information gained by the ordinary gelatin plate method. The property of liquefaction was formerly believed to be of significance, inasmuch as the liquefying bacteria were regarded as indicative of pollution. This position is, however, no longer tenable, since many bacteria, typical of the purest waters, may cause liquefaction.

CHAPTER X

THE SIGNIFICANCE AND APPLICABILITY OF THE BACTERIOLOGICAL EXAMINATION

Sanitary Inspection and Sanitary Analysis. The first attempt of the expert called in to pronounce upon the character of a potable water should be to make a thorough sanitary inspection of the pond, stream, well or spring from which it is derived. Study of the possible sources of pollution on a watershed, of the direction and velocity of currents above and below ground, of the character of soil and the liability to contamination by surface-wash are of supreme importance in interpreting the analyses to be made. In many cases, however, the results of the sanitary inspection will be found to be by no means conclusive. If house or barnyard drainage or sewage is actually seen to enter a water used for drinking purposes it is obviously unnecessary to carry out delicate chemical or bacteriological tests to detect pollution. On the other hand, no reconnoissance can show certainly whether unpurified drainage from a cesspool does or does not reach a given well; whether sewage discharged into a lake does or does not find its way to a neighboring intake; whether pollution of a stream has or has not been removed by a certain period of flow. Evidence upon

these points must be obtained from a careful study of the characteristics of the water in question, and this study can be carried out along two lines, chemical and bacteriological.

Sanitary Chemical Analysis. A chemical examination of water for sanitary purposes is mainly useful in throwing light upon one point—the amount of decomposing organic matter present. It also gives an historical picture which may be of much value. Humus-like substances may be abundant in surface-waters quite free from harmful pollution, but these are stable compounds. Easily decomposable bodies, on the other hand, must obviously have been recently introduced into the water and mark a transitional state. "The state of change is the state of danger," as Dr. T. M. Drown once phrased it. Sometimes the organic matter has been washed in by rain from the surface of the ground, sometimes it has been introduced in the more concentrated form of sewage. In any case, it is a warning of possible pollution, and the determination of free ammonia, nitrites, carbonaceous matter, as shown by "oxygen consumed," and dissolved oxygen yield important evidence as to the sanitary quality of a water.

Furthermore, nitrates, the final products of the oxidation of organic matter, and the chlorine introduced as common salt into all water which has been in contact with the wastes of human life, furnish additional information as to the antecendents of a sample. The results of the chlorine determination are indeed perhaps more clear than those of any other part of the analysis, for chlorine and sewage pollution vary together, due allow-

ance being made for the proximity of the sea and other geological and meteorological factors. Unfortunately, it is only past history and not present conditions which these latter tests reveal, for in a ground-water completely purified from a sanitary standpoint such soluble constituents remain, of course, unchanged. Thus, in the last resort, it is upon the presence and amount of decomposing organic matter in the water that the opinion of the chemist must be based.

Information Furnished by Bacteriological Examinations. The decomposition of organic matter may be measured either by the material decomposed or by the number of organisms engaged in carrying out the process of decomposition. The latter method has the advantage of far greater delicacy, since the bacteria respond by enormous multiplication to very slight increases in their food-supply, and thus it comes about that the standard gelatin-plate count at 20° roughly corresponds, in not too heavily polluted waters, to the free ammonia and "oxygen consumed," as revealed by chemical analysis. If low numbers of bacteria are found, the evidence is highly reassuring, for it is seldom that water could be contaminated under natural conditions without the direct addition of foreign bacteria or of organic matter which would condition a rapid multiplication of those already present. The bacteriologist in such cases can declare the innocence of the water with justifiable certainty. When high numbers are found the interpretation is less simple, since they may exceptionally be due to the multiplication of certain peculiar water forms. Large counts, however, under ordinary conditions,

when including a normal variety of forms indicate the presence of an excess of organic matter, derived in all probability either from sewage or from the fresh washings of the surface of the ground. In either case danger is indicated.

A still closer measure of polluting material may be obtained from the numbers of colonies which develop on litmus-lactose-agar at 37°, since organisms which thrive at the body temperature, and particularly those which ferment lactose, are characteristic of the intestinal tract and occur but rarely in normal waters.

Gage (Gage, 1907) has shown that by counts at 20, 30, 40, and 50° C., information may be quickly obtained which is of great assistance in judging the character of the water.

" Modern methods of bacterial examination of water, consisting usually of determinations of the numbers of bacteria by means of plates incubated at room temperature, and of tests for the presence or absence of one or two specific types, occasionally lead to an erroneous interpretation of the quality of a water, owing to the fact that they do not yield adequate data by which abnormal and inaccurate results may be separated from those which are truly indicative of purity or pollution. Furthermore, as several days must elapse before the bacterial tests can be completed, the results when obtained may have passed their usefulness. If, however, we can so modify our procedure that the varied character of the bacteria in waters of different classes may be quickly and accurately recognized, the value of bacterial water analysis will be enormously increased.

Much of this information may be obtained by the use of selective media, selective temperatures, or by a proper combination of the two.

"By the use of litmus-lactose-agar in place of agar or gelatin we obtain similar counts of total bacteria, and in addition are able to separate those bacteria into two groups, which do and do not produce acid fermentation of lactose, and the numbers of the two classes of bacteria so obtained indicate more completely the character of the water than would the numbers of either class alone. By incubating our plates at temperatures of 30 or 40° C. we are able to obtain counts in 12 to 18 hours, which counts, while smaller than those on plates incubated for a longer period at a lower temperature, appear to be fully as significant. If we increase our number of determinations by incubating duplicate plates at two or more temperatures, the various results and the ratios between them furnish a check upon one another in addition to increasing the available data upon which to base an interpretation." (Gage, 1907.)

Finally, the search for the Bacillus coli furnishes the most satisfactory of all single tests for fæcal contamination. This organism is preeminently a denizen of the alimentary canal and may be isolated with ease from waters to which even a small proportion of sewage has been added. On the other hand, it is never found in abundance in waters of good sanitary quality, and its numbers form an excellent index of the value of waters of an intermediate grade. The streptococci appear to be forms of a similar significance useful as yielding a certain amount of confirmatory evidence.

The full bacteriological analysis should then consist of three parts, the gelatin-plate count, as an estimate of the amount of organic decomposition in process; the total count, and the count of red colonies, on litmus-lactose-agar, as a measure of the organisms which form acids and thrive at the body temperature; and the study of a series of lactose bile tubes for the isolation of colon bacilli.

Special Advantage of the Bacteriological Examination. The results of the bacteriological examination have, in several respects, a peculiar and unique significance. First, this examination is the most *direct* method of sanitary water analysis. The occurrence of nitrites or free ammonia in a small fraction of one part per million, or of chlorine in several parts per million, do not in themselves render a water objectionable or dangerous. They merely serve as indicators to show that germ-containing and germ-sustaining organic matter is present. By a determination of the chlorine and study of the relations of carbon and nitrogen, it is possible to determine with some degree of accuracy whether this organic matter is of plant or animal origin, and hence to rate its objectionable or dangerous character. By the bacteriological examination, on the other hand, we are able to determine directly whether particular kinds of organisms characteristic of sewage are, or are not, actually present in the water. What we dread in drinking-water is the presence of pathogenic bacteria, mainly from the intestinal tract of man, and it is quite certain that the related non-pathogenic bacteria from the same source will behave more nearly

as these disease germs do than will any chemical compounds. In the second place, the bacteriological methods are superior in *delicacy* to any others. Klein and Houston (1898) showed by experiment with dilutions of sewage that the colon test was from ten to one hundred times as sensitive as the methods of chemical analysis; and studies of the self-purification of streams have confirmed their results on a practical scale. Thus in the Sudbury River it was found that while chemical evidences of pollution persisted for 6 miles beyond the point of entrance, the bacteria introduced could be detected for 4 miles further (Woodman, Winslow, and Hansen, 1902).

The statement is sometimes made that while bacteriological methods may be more delicate for the detection of pollution in surface-waters, contamination in ground-waters may best be discovered by the chemical analysis. That such is not the case has been well shown by Whipple (Whipple, 1903) who cites the following two instances in which the presumptive test revealed contamination not shown by the chemical analysis:

" A certain driven-well station was located in swampy land along the shores of a stream, and the tops of the wells were so placed that they were occasionally flooded at times of high water. The water in the stream was objectionable from the sanitary standpoint. The wells themselves were more than 100 feet deep; they penetrated a clay bed and yielded what may be termed artesian water. Tests for the presence of Bacillus coli had invariably given negative results, as might be naturally

expected. Suddenly, however, the tests became positive and so continued for several days. On investigation it was found that some of the wells had been taken up to be cleaned, and that the workmen in resinking them had used the water of the brook for washing them down. This allowed some of the brook-water to enter the system. It was also found that at the same time the water in the brook had been high, and because of the lack of packing in certain joints at the top of the wells the brook-water leaked into the suction main. The remedy was obvious and was immediately applied, after which the tests for Bacillus coli once more became negative. During all this time the chemical analysis of the water was not sufficiently abnormal to attract attention. On another occasion a water-supply taken from a small pond fed by springs, and which was practically a large open well, began to give positive tests for Bacillus coli, and on examination it was found that a gate which kept out the water of a brook which had been formerly connected with the pond was open at the bottom, although it was supposed to have been shut, thus admitting a contaminated surface-water to the supply." Whipple also calls attention to the report on the Chemical and Bacteriological Examination of Chichester Well-waters by Houston (Houston, 1901), in which the results of chemical and bacteriological examinations of thirty wells were compared. It was found that the bacteriological results were in general concordant and satisfactory. The wells which were highest in the number of bacteria showed also the greatest amount of pollution, as indicated by the numbers of B. coli, B. sporo-

genes, and streptococci. On the other hand, the chlorine and the albuminoid ammonia showed no correspondence with the bacteriological results.

Vincent (Vincent, 1905) cites an interesting case of the detection of progressive pollution of a ground-water by bacteriological methods. The well of a military camp in Algeria showed 200 bacteria per c.c. before the arrival of a regiment of troops. Its subsequent history is indicated in the table below:

PROGRESSIVE POLLUTION OF A WELL

(Vincent, 1905)

	Bacteria per c.c.	Bacillus coli per c.c.
Before arrival of troops	200	0
6 days after arrival	770	0
14 days after arrival	4,240	1
41 days after arrival	6,960	2
60 days after arrival	14,900	10

Thirdly, negative tests for Bacillus coli and low bacterial counts may be interpreted as proofs of the good quality of water, with a *certainty* not attainable by any other method of analysis. Many a surface-water with reasonably low chlorine and ammonias has caused epidemics of typhoid fever; but it is impossible, under any natural conditions (except perhaps in a well polluted with urine) that a water could contain the typhoid bacillus without giving clear evidence of pollution in the bile tube or on the lactose-agar plate.

In the examination of springs, especially those used for domestic supplies at country houses, the authors have found that the bacteriological examination offers a

more delicate and more certain index of the quality than may be obtained by chemical analysis. In a number of instances, springs located in pastures have become slightly polluted by animals, but to so small an extent that the chemical examination gave no indication of trouble. The bacteria, however, increased greatly in number, and colon bacilli could be readily isolated from 75 per cent of the 1-c.c. samples of a long series used in making the presumptive test. A single case may suffice as an illustration. This was a spring located on a hill in Hopkinton, Mass.

The chemical analysis was as follows:

Color	None
Turbidity	None
Sediment	None
Odor (hot)	None
Odor (cold)	None
	Parts per Million.
Total solids	33.0000
Loss on ignition	7.0000
Fixed residue	26.0000
Hardness	11.0000
Chlorine	10.0000
Nitrogen as—	
Albuminoid ammonia	0.0000
Free ammonia	0.0000
Nitrites	0.0000
Nitrates	0.0000

The bacteriological examination showed a total count of 375 bacteria per c.c. and a 37° count of 350 per c.c. The presumptive tests for Bacillus coli showed that gas-producing organisms were present in a majority of 1-c.c. samples, and typical colon bacilli were isolated. In this case the contamination was brought about by cattle gaining access to the area immediately surround-

ing the spring; but the same conditions might easily have led to infection from human beings.

Fromme (1910) cites several interesting examples of temporary pollution detectable only by bacteriological tests. The most striking case was that of an artesian well. Its average bacterial content had been 38 per c.c. and colon bacilli were absent from 200 c.c. In May, 1908, this well became polluted from a broken stable drain 10 meters away. The number of bacteria rose to 4370 and colon bacilli were found in 10 c.c. samples. The source of pollution was removed, but the well water in July still contained 7100 bacteria and B. coli in 1 c.c. In September the number had fallen to 105 and colon bacilli were present in 200 c.c. In November the bacteria numbered 120 and colon bacilli were absent from 200 c.c. At no time did chemical tests give any indication of danger, while the bacteriological data obviously measured very delicately a comparatively slight but real pollution and its gradual disappearance.

Similar results have been reported by Savage and Bulstrode (Savage, 1906) in the examination of the water-supply of Bridgend.

It seems to the writers that the real application of chemistry begins where that of bacteriology ends. When pollution is so gross that its existence is obvious and only its amount needs to be determined, the bacteriological tests will not serve, on account of their excessive delicacy. In studying the heavy pollution of small streams, the treatment of trades wastes, and the purification of sewage, the relations of nitrogenous compounds and of oxygen compounds are of prime

importance. In other words, when pollution is to be avoided, because the decomposition of chemical substances causes a nuisance, it must be studied by chemical methods. When the danger is sanitary and comes only from the presence of bacteria, bacteriological methods furnish the best index of pollution.

In the study of certain special problems the paramount importance of bacteriology is generally recognized. The distribution of sewage in large bodies of water into which it has been discharged may thus best be traced on account of the ready response of the bacterial counts to slight proportions of sewage, particularly since the ease and rapidity with which the technique of plating can be carried out make it possible to examine a large series of samples with a minimum of time and trouble. The course of the sewage carried out by the tide from the outlet of the South Metropolitan District of Boston was studied in this way by E. P. Osgood in 1897, and mapped out by its high bacterial content with greater accuracy than could be attained by any other method. Some very remarkable facts have been developed by similar studies as to the persistence of separate streams of water in immediate contact with each other. Heider showed that the sewage of Vienna, after its discharge into the Danube River, flowed along the right bank of the stream, preserving its own bacterial characteristics and not mixing perfectly with the water of the river for a distance of more than 24 miles (Heider, 1893). Jordan (Jordan, 1900), in studying the self-purification of the sewage discharged from the great Chicago drainage canal,

found by bacteriological analyses that the Des Plaines and the Kankakee Rivers could both be distinguished flowing along in the bed of the Illinois, the two streams being in contact, yet each maintaining its own individuality. Finally, the quickness with which slight changes in the character of a water are marked by fluctuations in bacterial numbers renders the bacteriological methods invaluable for the daily supervision of surface supplies or of the effluents from municipal filtration plants.

In the commoner case, when normal values obtained by such routine analyses are not at hand, the problem of the interpretation of any sanitary analysis is a more difficult one. The conditions which surround a source of water supply may be constantly changing. No engineer can measure the flow of a stream in July and deduce the amount of water which will pass in February; yet the July gauging has its own value and significance, so a single analysis of any sort is not sufficient for all past and future time. If it gives a correct picture of the hygienic condition of the water at the moment of examination it has fulfilled its task, and this the bacteriological analysis can do. The evidence furnished by inspection and by chemical analysis should be sought for and welcomed whenever it can be obtained, yet we are of the opinion that, on account of their directness, their delicacy, and their certainty, the bacteriological methods should least of all be omitted.

CHAPTER XI

BACTERIOLOGY OF SEWAGE AND SEWAGE EFFLUENTS

Bacteriological and Chemical Examination of Sewage. The first object of modern sewage disposal is the oxidation of putrescible organic matter. Chemical, rather than bacterial, purification is usually the prime requisite; and chemical tests therefore serve best as criteria of the results obtained. Bacteria are the agents in the process of sewage purification; but the most generally useful measure of the work accomplished is the chemical oxidation attained. "To employ a simile, it is a case of the saw and the 2-foot rule—the saw will do the cutting, but the rule will measure the work cut." (W. J. Didbin.)

In certain cases, however, bacterial as well as chemical purity must be effected, in view of special local requirements. The sewage from a contagious disease hospital, for example, should be freed from infectious material as a factor of safety. Sewage discharged into a body of water adapted for bathing may well be so treated as to protect those using the water. In the case of seaboard cities where sewage effluents are likely to contaminate oyster beds and other layings of edible shellfish the problem assumes great importance. Where bacterially impure effluents are discharged into streams used for

sources of water-supply the town taking water may protect itself by filtration. It should so protect itself, at any rate, from the pollution necessarily incident to surface waters; and, unless the bacterial condition of a stream or lake is made very materially worse by the discharge of sewage effluents, it is fair that the responsibility of purification should rest on the water works, rather than on the sewage purification plant. Shellfish, on the other hand, cannot be purified. Either pollution must be prevented, or the industry abandoned. Under such circumstances sanitary authorities may rightly demand, as they have demanded at Baltimore, that bacteria, as well as putrescible organic matter, shall be removed in sewage treatment. Under such circumstances the bacterial control of purification plants is as essential as in the case of water filters.

Methods of Bacteriological Examination of Sewage and Effluents. In England, considerable attention has been devoted to this subject, and numerous methods have been recommended as furnishing valuable criteria of the bacterial quality of sewage effluents. Houston (1902[b]), for example, suggests various tests involving the use of litmus milk, peptone solution, gelatin tubes, and neutral-red broth, as well as the inoculation of animals. He considers the determination of the numbers of B. coli and B. sporogenes as of greatest moment, while the identification of streptococci is of value in certain cases and the enumeration of liquefying bacteria, spore-forming aerobes, thermophilic bacteria, and hydrogen sulphide producing bacteria is of subsidiary importance. Rideal (1906) has recently recommended a some-

what less extensive series of tests, including aerobic and anaerobic counts, both at 20 and 37°, with the determination of the number of liquefiers and the number of spore-formers. The results attained do not seem to warrant any such elaborate procedure. As far as the authors are aware, the determination of liquefying bacteria, anaerobic bacteria and thermophilic bacteria does not add any information of material importance to that obtained from the total count. Some test for specific sewage organisms is of course desirable. Here again, however, the determination of B. sporogenes and sewage streptococci tells the observer little more than can be learned from the routine use of the colon test. In the United States the practise of sewage bacteriologists is crystallizing around the total count and the estimation of B. coli. In the absence of evidence as to the specific value of other data, the routine control of filter plants may well be limited to these two determinations.

The total count of bacteria should be made, as in the case of waters, at 20°. Determinations carried out in duplicate at 37° give additional information of considerable value. The ratio of the 37° count to the 20° count varies with different sewages. At Boston the body temperature count is 70 to 80 per cent of the total count; at Lawrence it appears to be proportionately much lower (Gage, 1906). In using either medium, it is well to add lactose and litmus and note the number of red colonies, as a check on the enumeration of B. coli.

It should be borne in mind, as Lederer and Bach-

mann (1911) have recently pointed out, that the sampling error is a very serious one with sewage. Duplicate tests made at 1-minute intervals for a period of 10 minutes in their experiments gave extreme values of 190,000 and 550,000 per c.c.

The determination of the number of colon bacilli in sewage and effluents should furnish an integral part of bacteriological sewage analysis, since it is important to know whether the decrease of intestinal bacteria in the process of purification is proportional to the reduction of total bacteria. The State Sewerage Commission of New Jersey has adopted this procedure in its supervision of the disposal plants in that State; and the results seem amply commensurate with the labor involved. As in the case of polluted waters the enumeration of B. coli may be carried out, either by the study of the red colonies which appear on litmus-lactose-agar plates inoculated with the sample directly, or by the use of a preliminary enrichment process. The complete identification of B. coli seems unnecessarily tedious, however, where the organisms are present in such abundance. Some approximate presumptive method is indicated here, if anywhere; and the experience with polluted water, reviewed in Chapter VI, points to the Jackson bile medium as the most promising one. Experience at the Sewage Experiment Station of the Massachusetts Institute of Technology has shown that this presumptive test in general yields good results. As pointed out above, a 48-hour incubation period at 37° is required. All tubes showing 20 per cent gas at the end of this time may be con-

sidered positive tests for the colon group, without serious error.

Numbers of Bacteria in Sewage. The total number of bacteria and the number of colon bacilli naturally vary widely in the sewages of different cities and towns. European sewages, being more concentrated, show as a rule higher numbers than are found in America. Results compiled from various sources show from 1,000,000 to 5,000,000 bacteria in the sewages of Essen, Berlin, Charlottenburg, Leeds, Exeter, Chorley, and Oxford, 2,000,000 to 10,000,000 in the sewages of London, Walton, and W. Derby and over 10,000,000 in the sewages of Paris, Ballater and Belfast (Winslow, 1905). The number of colon bacilli in English sewages varies from 50,000 to 750,000. In American sewages, on the other hand, bacteria are somewhat less numerous. At Lawrence the determinations made from 1894 to 1901 showed on the average 2,800,000 bacteria per c.c. At Worcester, Eddy reported 3,712,000 in 1901 (Eddy, 1902); at Ames, Iowa, Walker (1901) found 1,248,256 in the same year. At Columbus, Johnson (1905) reports an average of 3,600,000 bacteria per c.c.; the individual numbers varied from 320,000 to 27,000,000. The number of colon bacilli varied from 50,000 to 1,000,000 and averaged 500,000. Day samples of Boston sewage collected three times a week, from October, 1906, to April, 1907, showed an average of 1,200,000 bacteria per c.c. In the summer months numbers are notably higher than at other seasons in many sewages. Thus in 1903, Boston sewage contained 2,995,000 bacteria in July, 4,263,600 in August,

11,487,500 in September, 3,693,000 in October, 587,100 in November, and 712,000 in December (Winslow, 1905). There is also a marked diurnal variation in the bacterial content of sewage, since the flow contains a smaller proportion of intestinal matter at night than at other times. For example, a series of hourly samples at the Sewage Experiment Station of the Massachusetts Institute of Technology showed the following results:

BACTERIA IN BOSTON SEWAGE—AVERAGES FOR EACH FOUR-HOUR PERIOD. AUGUST 13–14, 1903

(Winslow and Phelps, 1905)

Period.	Bacteria per c.c.
7:30–11:30 A.M.	1,800,000
11:30 A.M.–3:30 P.M.	3,200,000
3:30–7:30 P.M.	4,600,000
7:30–11:30 P.M.	3,500,000
11:30 P.M.–3:30 A.M.	1,000,000
3:30–7:30 A.M.	400,000

It is evident that many published results of bacterial examinations of sewage are in excess of the average values, since they refer in most cases to day samples only.

Bacterial Content of Sewage Effluents. The bacterial content of sewage effluents varies widely according to the process of purification adopted and the efficiency of the particular plant. The only process which yields a notably purified effluent from the bacteriological standpoint is that of filtration through sand. Processes of this type when operated with care may give a bacterial purification well over 99 per cent as

shown by bacteriological examinations at the Brockton (Mass.) filters, reported by Kinnicutt, Winslow and Pratt (1910) as follows:

BACTERIA IN SEWAGE AND EFFLUENTS AT BROCKTON, AVERAGE OF FOUR EXAMINATIONS, AUTUMN OF 1908

	Bacteria per c.c. Gelatin 20°.	Colon Bacilli per c.c. Lactose Bile.
Sewage	3,150,000	150,000
Effluent A	1,900	400
" B	6,300	15
" D	125	0
" E	1,400	5
" F	2,000	1

Such high efficiencies as this table indicates are often not realized under the actual working conditions of a municipal plant. At Vineland, N. J., for example, the intermittent filters show a reduction of 90 to 95 per cent in total bacteria and a somewhat higher reduction of B. coli. The results of three examinations made in 1906 are given below.

BACTERIA IN SEWAGE AND SAND FILTER EFFLUENT AT VINELAND, N. J.

(N. J. State Sewerage Commission, 1907)

Date.	Bacteria per c.c.		B. Coli in	
	Sewage.	Effluent.	Sewage.	Effluent.
March 2	480,000	20,000	0.0001 c.c.	0.01 c.c.
July 26	496,000	61,000	0.0001 c.c.	0.001 c.c.
July 26	511,000	38,000	0.00001 c.c.	0.001 c.c.

The newer bacterial processes, contact beds, and trickling filters naturally show a much less satisfactory bacterial removal than sand filtration beds. In the Columbus experiments, Johnson (1905) found from 1,000,000 to 2,000,000 bacteria in the effluents of contact beds and from 750,000 to 1,900,000 in the effluent from trickling filters.

At the experiment station of La Madeleine, in Lille, Calmette (1907), reports 5,000,000 bacteria per c.c. in the crude sewage, 2,900,000 in the second contact effluent and 800,000 in the effluent from the trickling bed. Of 20,000 B. coli per c.c. applied to the filters, the contact system delivered 4000 and the trickling bed 2000 per c.c. The average results of examinations made three times a week at the Sewage Experiment Station of the Massachusetts Institute of Technology, during two different periods, were as follows:

BACTERIA IN SEWAGE, SEPTIC EFFLUENT AND TRICKLING EFFLUENT AT BOSTON

(Winslow and Phelps, 1907)

	Bacteria per c.c.				B. Coli. Positive Tests in 0.000001 c.c.*
	July–Sept., 1906.		Oct., 1906–April, 1907.		July–Sept., 1906.
	No.	Per Cent Reduction.	No.	Per Cent Reduction.	Per Cent.
Sewage	1,300,000	.....	1,200,000	....	65
Septic effluent	1,650,000	Inc.	750,000	38	66
Effluent from trickling bed	750,000	42	200,000	83	35
Septic tank and trickling bed	750,000	42	180,000	85	35

* Jackson bile test.

The following average data for two of the largest trickling filter plants in the United States are cited by Kinnicutt, Winslow and Pratt (1910).

BACTERIAL CONTENT OF SEWAGE AND EFFLUENTS FROM TRICKLING FILTERS

Place.	Period.	Bacteria per c.c.		
		Screened Sewage.	Septic Effluent.	Filter Effluent.
Reading, Pa.....	1908–1909	3,100,000	1,800,000	600,000
Columbus, Ohio...	1909	2,370,000	1,050,000	560,000

It is obvious that effluents of this character cannot be considered satisfactory from the standpoint of bacterial purification. As Houston concluded, after a careful review of the subject, " The different kinds of bacteria and their relative abundance appear to be very much the same in the effluents as in the crude sewage. Thus, as regards undesirable bacteria, the effluents frequently contain nearly as many B. coli, proteus-like germs, spores of B. enteritidis sporogenes and streptococci, as crude sewage. In no case, seemingly, has the reduction of these objectionable bacteria been so marked as to be very material from the point of view of the epidemiologist " (Houston, 1902[a]).

Experimental studies with specific bacteria have confirmed these conclusions. Houston (1904[b]) found that B. pyocyaneus appeared in the effluent of a trickling bed 10 minutes after application to the top and continued to be discharged for 10 days. In septic tanks and contact beds, the same germ persisted for 10 days.

Rideal (1906) quotes experiments by Pickard at Exeter, which show that typhoid bacilli may persist for 2 weeks in a septic tank and that contact bed treatment only effects a 90 per cent removal of these organisms.

Disinfection of Sewage Effluents. Where bacterial purity is required, some special process of disinfection must be combined with the contact bed or the trickling filter. For this purpose treatment with chloride of lime or other chemicals is rapidly gaining ground as an important adjunct to bacterial disposal plants; and in connection with this process bacteriological control is an essential.

Rideal (1906) first showed at Guildford that 30 parts of available chlorine per million would reduce the number of bacteria in crude sewage from several millions to 50,000, while 50 parts would reduce their number to 20 per c.c. Colon bacilli were reduced from one million per c.c. to less than one per c.c. by 30 parts of chlorine. In septic effluent 25 to 44 parts of chlorine per million reduced B. coli from two and a half to four and a half million per c.c. to less than one per c.c. With contact effluents smaller amounts of chlorine proved efficient, The primary effluent required 20 parts per million, the secondary effluent 10.6 parts per million and the tertiary effluent 2.5 parts per million to reduce the number of B. coli so that this organism could not be isolated in 5 c.c.

In this country Phelps and Carpenter (1906) demonstrated the practical usefulness of bleaching powder disinfection, at the Sewage Experiment Station of the Massachusetts Institute of Technology. As indicated

in the table below smaller amounts of chlorine than were used by Rideal will give good results with more dilute American sewages.

BACTERIA IN TRICKLING FILTER EFFLUENT BEFORE AND AFTER TREATMENT WITH CHLORIDE OF LIME (5 PARTS PER MILLION AVAILABLE CHLORINE)

(PHELPS AND CARPENTER, 1906)

Date.	Bacteria per c.c.		B. Coli, Jackson Bile Test.	
	Before.	After.	Before 0.000001 c.c.	After 1.0 c.c.
1906				
August 11....	270,000	69	+ o	+ o
" 13....	630,000	41	o o	+ o
" 14....	135,000	406	+ +	+ o
" 15....	230,000	21	o o	o o
" 16....	250,000	37	+ o	o o
" 18....	110,000	40	o o	+ o
" 20....	90,000	54	+ o	o o
" 21....	220,000	22	o o	o o
" 23....	.....	.....	+ o	o o
Average.....	240,000	86	33%+	22%+
Average removal....	99.96%		99.993%	

The success of chemical disinfection varies with the character of the sewage or effluent treated, since the organic matter present consumes a certain amount of the disinfectant and renders it inoperative. Discordant results are therefore reported from different sources.

An important series of experiments carried out in Ohio by Kellerman, Pratt, and Kimberly (1907) showed good results with sand filter effluents and

contact effluents. Septic sewage, on the other hand, required large amounts of chlorine to produce a reasonable bacterial reduction. The table on page 240 shows the results obtained at Marion, Ohio.

In Germany, on the other hand, Schumacher (1905), Kranepuhl (1907), and Kurpjuweit (1907) found larger amounts of chlorine necessary, in the neighborhood of 60 parts per million parts of sewage. Their tests were somewhat severe, however, the criterion of success being the absence of B. coli in a large proportion of liter samples.

Standards for Sewage Effluents. The science of sewage bacteriology is in its infancy; and it is difficult to give any general rules for the interpretation of bacteriological examinations designed to indicate whether disposal plants are successful or not. Houston stated provisionally that the 20° count should be under 100,000 and the 37° count under 10,000, while B. coli should be absent from .001 c.c. and B. sporogenes from .1 c.c. (Houston, 1902[b]). This standard seems to us far too lenient. Either organic purity alone is necessary, as at many sewage disposal plants, or a higher grade of purity than this should be attained. It seems wisest at the present time to avoid fixing any general standards of purity for sewage effluents. Each case should be judged intelligently on its own merits. In general, however, where bacterial purification is indicated at all, it seems fair to demand that the effluent should be of such a quality as not to increase materially the bacterial content of the body of water into which it is discharged.

BACTERIA IN SEPTIC EFFLUENT, CONTACT EFFLUENT, AND SAND EFFLUENT AT MARION, O., BEFORE AND AFTER TREATMENT WITH CALCIUM HYPOCHLORITE

(KELLERMAN, PRATT, AND KIMBERLY, 1907)

Date.	Effluent.	Average Available Chlorine. Parts per Million.	Bacteria per c.c.			
			20° C.		37° C. Total Count.	
			Untreated.	Treated.	Untreated.	Treated.
1907						
Apr. 11	Septic	4.3	850,000	1,100,000	1,200,000	240,000
Apr. 12	Septic	6.2	4,400,000	550,000	850,000	260,000
Apr. 15	Septic	7.6	600,000	400,000	450,000	190,000
Apr. 28	Contact	2.9	110,000	2,500	.....	.....
Apr. 29	Contact	5.0	65,000	1,600	73,000	370
Apr. 30	Contact	4.4	500,000	800	160,000	400
Mar. 21	Sand	3.8	49,000	570	9,800	150
Mar. 22	Sand	3.0	56,000	140	7,000	60
Mar. 26	Sand	1.5	70,000	4,000	20,000	160

Date.	Effluent.	Average Available Chlorine. Parts per Million.	Bacteria per c.c.			
			37° C. Red Colonies.		B. Coli.	
			Untreated.	Treated.	Untreated.	Treated.
1907						
Apr. 11	Septic	4.3	55,000	7,400		
Apr. 12	Septic	6.2	60,000	15,000		
Apr. 15	Septic	7.6	100,000	51,000		
Apr. 28	Contact	2.9	.....	.....	20,000	Not in 0.5
Apr. 29	Contact	5.0	10,000	0	15,000	" 0.5
Apr. 30	Contact	4.4	21,000	3	20,000	" 1.0
Mar. 21	Sand	3.8	1,300	0	1,000	" 1.0
Mar. 22	Sand	3.0	800	0	2,000	" 1.0
Mar. 26	Sand	1.5	4,000	1	2,000	In 1.0

Bacteriology of the Sewage Filters Themselves. Before leaving the subject of sewage bacteriology, brief reference must be made to the importance of bacteriological studies in relation to the processes of sewage purification which bring about the removal of the organic matter itself. Nothing is more necessary to the development of the present art of sewage disposal than knowledge of the micro-organisms concerned and of the conditions which favor their activity; but such knowledge is woefully deficient. Something is known of the nitrifying organisms long ago discovered by Winogradsky. More recent work, like that of Schultz-Schultzenstein (1903), Boullanger and Massol (1903) and Calmette (1905), has cleared up many points concerning these forms; but much remains to be done. In regard to the reducing action of bacteria in the septic tank and contact bed we are almost wholly in the dark. Septic tanks work well with some sewages and badly with others; and the presence or absence of the right bacteria is probably largely responsible for the different results. In some cases, as at Plainfield, N. J., the seeding of a tank with cesspool contents has produced a material improvement in septic action.

Knowledge of the kinds of bacteria involved would make it possible to substitute scientific control for such empiricism and might well lead to improved methods of a more intensive character than are yet available. The work already done upon a laboratory scale furnishes promise of such results. The student

who wishes to follow out this line of investigation will find a good summary of what is already known of the hydrolysis and denitrification of nitrogenous bodies and the decomposition of cellulose and other carbohydrates in Rideal's "Sewage and the Bacterial Purification of Sewage" (1906).

Gage (1905) has made a suggestive study of the bacteria which carry on the reducing changes in sewage which deserves the attention of all who are interested in the more theoretical aspects of sewage treatment. His method consisted in plating sewages and effluents, isolating typical cultures and determining their power to decompose peptone and nitrates with the production of ammonia and free nitrogen. The rate of gelatin liquefaction, the amount of nitrate reduced, the amount of free ammonia formed, and the amount of nitrogen liberated were quantitatively determined for each culture thus isolated. The numerical values obtained, multiplied by the number of bacteria, apparently of the same type, observed in the plates, gave coefficients of the liquefying, denitrifying, ammonifying, and nitrogen-liberating power of the effluent; and these coefficients may be considered as measures for a given sample of the tendency of the bacterial flora to set up certain changes. The results of further studies made by Clark and Gage (1905), on sewages and on sand, contact, and trickling effluents, show that there may be important differences between various sewages in this respect which must render their purification more or less easy. They indicate that the effluents obtained from intermittent sand

filters in cold weather contain larger numbers of ammonifying and denitrifying bacteria than appear at other seasons, which may help to explain the poorly nitrified effluents obtained in the winter season. Along these lines research work in sewage bacteriology promises to be fruitful of results.

CHAPTER XII

BACTERIOLOGICAL EXAMINATION OF SHELLFISH

Shellfish and Disease. The pollution of areas devoted to the growing of shellfish and the consequent pollution of the shellfish themselves is a matter of much sanitary importance. Oysters, clams and mussels are the shellfish commonly used as food, and since they are likely to be eaten in an uncooked or partially cooked condition, it is important to be assured as to their character from the bacteriological standpoint. In their normal habitats, in clean sea-water, or in river estuaries free from pollution, shellfish are unquestionably free from dangerous bacteria, although their feeding habits make it probable that the types of bacteria indigenous to the waters in which they are found might be present in considerable numbers. With the pollution of streams by unpurified sewage the areas in which oysters and clams develop may easily become infected by organisms of intestinal types, and there is, therefore, offered an easy means for the typhoid bacillus and other pathogenes to pass from the sewage directly into the intestinal tract of the consumer of the raw oysters or clams.

The history of this subject is well summarized by Newlands and Ham (1910), from whose excellent report the following paragraphs are adapted:

Attention was first drawn to the danger from shellfish by the remarkable outbreak of typhoid fever which occurred in Middletown, Conn., in 1894, as a result of the serving of raw oysters at college fraternity banquets. The oysters used in this case were all derived from a certain portion of Long Island Sound, where they had been put down, or planted, in order to fatten. Investigation showed that the stream entering the Sound at this point was highly polluted, and furthermore, that at a nearby house there were two severe cases of typhoid fever from which the intestinal discharges were turned into the drain and thence into the stream without disinfection. The course of the passage of the bacteria from the patient suffering with the disease to the oyster and so on to the young men at the banquets was, therefore, traced out in a most complete and thorough way. This investigation, which was conducted by Prof. H. W. Conn, of Wesleyan University, caused immediate investigations to be set on foot in England and in this country. Two years later there followed a report by the Local Government Board of Great Britain dealing with pollution of shellfish along the English coast, and the matter has also received much attention in this country.

A study of the literature reveals only a few references to oysters as carriers of disease germs previous to 1880. In that year Cameron, in a paper entitled "Oysters and Typhoid Fever," read before the British Medical Association, suggested that outbreaks of typhoid fever and cholera might be caused by eating oysters.

In 1893 Thorne-Thorne, in a report to the Local Government Board, wrote that, in his opinion, certain cases of cholera which had occurred that year at various inland towns in England were due to eating contaminated oysters from beds at Grimsby, where there had been a small cholera epidemic. Following the suggestions embodied in this report the English Government began a series of investigations which have made many important additions to our present knowledge of the subject.

In 1902 the famous oyster epidemics at Winchester and Southampton, England, were proven beyond reasonable doubt to have been caused by contaminated oysters taken from grounds at Emsworth. Here again we have to deal with banquets given in different cities where the only common source of infection appears to have been contaminated oysters. Of the 267 guests at these banquets 118 were attacked with intestinal disorders and 21 cases of typhoid fever developed, 5 of which were fatal.

Although a great many sensational attacks have been made against oysters as carriers of disease germs which have been based on little or no evidence, the above-mentioned investigations and others, among which might be mentioned those of Thresh, Marvel, and Soper, have brought out sufficient trustworthy evidence to show that contaminated oysters must be considered as a real factor in the dissemination of typhoid fever and other water-borne diseases. An estimate of the extent to which such illness is due to oysters would be impossible at the present time. The

Royal Sewage Commission after an extensive investigation on this subject came to the following conclusion: "After carefully considering the whole of the evidence on this point, we are satisfied that a considerable number of cases of enteric fever and other illness are caused by the consumption of shellfish which have been exposed to sewage contamination; but in the present state of knowledge, we do not think it possible to make an accurate numerical statement.

"Moreover an examination of the figures which have been placed before us as regards those towns in which the subject has been most carefully studied shows that there may be occasional errors. Indeed the witnesses themselves recognized that absolutely accurate figures were not obtainable.

"We are far from denying that isolated cases may have been due to contaminated shellfish, but we must remember that the possibility of some of them being due to other causes cannot be altogether excluded."

In the above-mentioned cases, where oysters have been proven or reasonably suspected of being the cause of disease, it was found that the oysters in question had been floated or grown in heavily polluted water where direct contact with specific infection could be proven or readily assumed. The Wesleyan epidemic is a case in point. Oysters had undoubtedly been floated in the contaminated waters at Fair Haven for a number of years previous to 1894 without any noticeable effect on the health of persons eating them, but specific infection of the water from two patients in a house near by was followed by a serious epidemic.

Valuable studies of the relation between shellfish and disease have recently been published by Bulstrode (1911) and Wilhelmi (1911) and Stiles (1912).

Effect of Cookery upon Polluted Shellfish. It should be noted that it is unfortunately not only raw shellfish which are responsible for the spread of disease. Most of the processes of cookery to which these foods are subjected are insufficient to destroy pathogenic germs. Clark (1906) found that clams and oysters in stews and fried and scalloped in the usual manner were generally free from colon bacilli and streptococci. With steamed clams, however, the bacteria present could not be destroyed except by a temperature high enough and prolonged enough to ruin the clams for eating. Rickards (1907) confirmed these results as to the danger from steamed clams, while he found fried clams and clams in chowder and scalloped oysters to be practically sterilized. Oyster stew, however, is not exposed to long continued heat as is clam chowder, and fried oysters are less thoroughly heated than fried clams in the ordinary processes in use. Oysters in both of these forms and fancy roast oysters still contained colon bacilli and streptococci. Buchan (1910) finds that the ordinary methods of cooking mussels do not remove the risk of typhoid infection.

Bacteriological Examination of Shellfish. Without further discussing the general sanitary aspects of the subject it is important to consider just how one may determine whether the oysters from a given region are polluted or not. The methods which have been developed for this work are essentially modifications

of the methods used in water examination, involving sometimes total counts of bacteria at different temperatures, but especially the application of the various tests for the determination of the colon bacillus, since here, as in water examination, this organism may be taken as an index of pollution and its occurrence in considerable numbers must be looked upon not merely with suspicion, but as a practical proof that the supernatant waters are polluted and that the shellfish themselves may contain organisms of pathogenic importance, such as B. typhi, B. dysenteriæ, B. sporogenes and others. Determinations of the pollution of the water above the beds are sometimes made as bearing indirectly and inferentially on the possibility of the pollution of the shellfish contained therein. Results of the two determinations are not always in close agreement, however, owing to the rapidly changing local conditions due to tide, etc. The general relations and the individual variations between water and shellfish determinations are well illustrated in the table on page 250 from the report by Newlands and Ham (1910) on conditions in New Haven Harbor.

Study of the methods of examination of shellfish has been conducted with great care at the Lawrence Experiment Station by Gage, at the Sanitary Research Laboratory at the Institute of Technology by Phelps, at Brown University by Gorham, and in New York by Pease. Other officials of the Shellfish Commissions of different States have also carried out investigations upon this subject. The Lawrence Experiment Station

BACTERIA IN WATER AND SHELLFISH, NEW HAVEN HARBOR

Station.	Water.				Oysters.		
	Samples Taken.	Av. Number Bacteria per c.c.		Average Number B. Coli * per c.c.	Average Number B. Coli * per c.c.	Number Oyster Samples	Character of Bottom.
		37° C.	20° C.				
Ferry St. Bridge	12	210	1260	43	.....	.....	Soft
Tomlinson Br.	15	910	2650	34	.....	.....	"
No. 1...	15	510	1680	51	.....	.....	"
No. 2...	15	375	910	73	.....	.....	"
No. 3...	16	255	835	9	72	16	"
Buoy 10.	15	155	450	10	.....	.....	"
No. 4...	15	160	1720	9	.....	.....	"
No. 5...	17	615	1340	74	308	13	"
Buoy 5..	23	315	715	15	.....	.....	"
Buoy 8..	15	205	410	8	.....	.....	"
No. 6...	16	145	485	8	37	6	Seaweed
No. 7...	21	215	740	29	425	11	Hard
No. 9B..	11	220	260	7	64	10	"
No. 9A..	13	100	185	9	46	10	"
No. 9...	12	195	200	17	37	10	"
No. 7A .	11	120	240	10	255	11	"
No. 8...	16	180	270	7	370	6	
No. 10..	23	300	615	9	100	1	Soft
No. 11..	11	405	510	8	10	4	"
Buoy 6..	21	815	1690	9	.....	.....	
Buoy 3..	17	175	590	6	291	8	Hard
No. 12..	14	620	1190	4	6	5	"
No. 13..	7	240	120	10	10	8	"
No. 14..	12	285	1100	1 —	7	8	"
Buoy 4..	7	375	1400	4			
No. 15..	7	455	1680	1	45	15	Hard
No. 16..	14	280	1025	1 —	1 —	3	Soft
No. 17..	14	300	1260	1 —	.....	.....	Hard
No. 19..	1	800	300				
No. 20..	10	135	860	1 —	10	8	Hard
Buoy 2..	11	375	905	2			
No. 22..	8	305	500	1 —	7.3	3	Hard
Buoy 1..	6	115	995	1 —	4	12	"
No. 18..	10	255	675	1 —			
No. 24..	4	710	1340	4	9	10	Hard
No. 23..	6	450	240	1 —			"
No. 25..	2	130	1000	1 —			
No. 26..	5	130	465	1 —			
No. 27..	10	630	695	1 —	.....	.....	Soft
No. 28..	4	415	1400	1 —	4	3	Mud and sand
No. 29..	5	370	1700	1 —	.....	.....	Soft
No. 30..	5	185	440	1 —	1 —	15	Hard
No. 31..	7	320	130	1 —	1 —	15	"
No. 32..	5	70	1050	1 —	3	15	"
No. 33..	4	485	405	1 —	1 —	10	"
No. 34..	4	535	495	1 —	1	10	"
No. 35..	4	120	270	1 —	1	15	Sticky

* Jackson's lactose bile presumptive test used.

Minus sign after figure 1 indicates that the average was less than 1.

method was published in the Massachusetts State Board of Health Report for 1905 (Clark, 1906). This method consisted in the total counts of bacteria developing at 20° and 37° and the fermentation reaction in dextrose broth. Experience indicated that it was not merely necessary to examine the stomach contents of the oysters but the "shell water" as well was subjected to examination. With the advent of lactose bile as a better medium for the development of B. coli without interference with other types of bacteria, the substitution of this medium for dextrose broth was commonly made, and this is now one of the standard media employed for the determination.

It has been noted that the superiority of lactose bile to dextrose broth is greatest in water examinations when the water is most polluted. In the study of shellfish the danger of overgrowths is even greater than in polluted waters, since the organic matter in the oyster and its surrounding shell water furnishes a culture medium for many bacteria. Streptococci are particularly abundant. As pointed out in Chapter IX, streptococci die out more rapidly than colon bacilli in potable waters, but where organic matter is present in abundance the former may survive the latter.

We have compiled the table on page 252 from the results of Clark (1906). In all cases except in that of the shell water there is a considerable difference between the dextrose fermentation tests and the colon isolations, indicating an overgrowth by streptococci and other forms, of colon bacilli originally present. The B. sporogenes is also very frequently

responsible for such anomalous results in shellfish examinations.

COLON BACILLI AND STREPTOCOCCI IN DIFFERENT PORTIONS OF THIRTY CLAMS

	Per Cent of Samples Showing			
	Fermentation in Dextrose Broth.	B. Coli.	Streptococci.	B. Coli and Streptococci.
Shell water	90	83	47	40
Gills	77	53	25	15
Stomach (intestine)	55	35	22	12
Rectum (intestine)	82	45	43	13
Liver	37	18	15	3
Visceral tissue	18	8	7	2

It will be noted from Clark's table above that the shell liquor is not only freer from overgrowths than the portions of the body of the clam, but that the proportion of positive reactions is in each case higher. Since the shell water is of course easier to examine than the macerated animal, this is now generally adopted as the standard material for examination.

Self-purification of Shellfish. In connection with the bacteriological examination of shellfish for colon bacilli certain investigations have been carried out which are of great importance from the commercial as well as from the sanitary standpoint. Phelps (1911) has shown that oysters which develop in waters subject to sewage pollution may be purified or entirely freed from colon bacilli by the removal of the oysters themselves to waters of purer character, when, after

sufficient time has elapsed, the oysters will have cleansed themselves through their metabolic processes and become entirely safe even for consumption in the raw state. It is of considerable importance to determine the length of time necessary for this self-purification to take place. Obviously, from the commercial standpoint it is desirable to make it as short as possible, while from the sanitary standpoint it must be long enough to insure a thorough and satisfactory removal of all traces of polluted matter. Oyster beds which are free from pollution or which are sufficiently good for the re-laying for polluted oysters are difficult to find and limited in areas because of their nearness to sources of pollution. The investigations in question were conducted by Phelps in the Providence River and the upper part of Narragansett Bay. The oysters were removed from heavily polluted regions and carried to waters which were practically free from pollution, where they were planted. Examinations were made from day to day in order to determine the length of time that these particular oysters showed pollution and it was found that within 4 days the organisms of the colon type were practically all eliminated.

It must be borne in mind that, if shellfish are carelessly opened and handled, they may suffer a considerable additional pollution in the process, and may therefore be much worse instead of better than when they were taken. This is well brought out by the table on page 254, taken from a report by Stiles (1911) in which shucked market oysters show much worse pollution than market oysters in the shell.

COMPARATIVE BACTERIOLOGICAL CONDITION OF MARKET OYSTERS, SHUCKED AND IN THE SHELL

(STILES, 1911).

	Number of Samples.	Average per c.c. Liquor.				
		Bacteria.			B. Coli.	Streptococci.
		Plain Agar.		Bile Salt Agar.		
		25°	37°	37°		
Shell oysters.....	36	6,000	1,000	200	7	....
Shucked oysters..	33	867,000	268,000	45,000	74,000	8000

Seasonal Variation of Bacteria in Oysters. It has been observed by Gorham (1912) and others that the examination of oysters from certain regions made in the summer failed to agree with the similar analyses from the same beds made in the winter. With the advent of cold weather there seems to be a great improvement in the sanitary quality, so that oysters taken from beds in close proximity to the outfalls of large sewers show in the colder months entire absence of any evidence of contamination, judged solely by the bacteriological data. Thus Gorham found in the summer of 1910 that all oysters on the beds in the Providence and Warren Rivers and the upper part of Narragansett Bay were so badly polluted by sewage as to be unfit for food. Colon bacilli were found in the "shell water" of every oyster in amounts as small as .01 of a cubic centimeter or less. Chemical and bacteriological examination of the waters over these

beds showed them to be heavily sewage polluted. In December of the same year the analyses of the oysters were strikingly different; although the condition of the water was apparently unchanged. In the examination five oysters were selected in each case and the average total number of bacteria per cubic centimeter was determined and the presence of colon bacilli was tested by the bile tube and subsequent isolation and identification of the organisms. The table on page 256 shows the numbers of bacteria found, and the proportion of the five oyster samples in which colon bacilli were present in cubic centimeter amounts and also in 0.1 and 0.01 of a cubic centimeter.

The conclusions arrived at by Gorham are that during the cold weather the oysters assume a condition of rest or hibernation, during which time ciliary movement ceases and the process of feeding is suspended. No organisms are therefore taken in from the outside water and those inside the oyster are gradually eliminated, so that the total number of organisms is reduced very considerably and the oyster becomes practically free from colon bacilli.

Standard Methods for the Examination of Shellfish. The examination of shellfish for pollution is regarded as of such importance by the American Public Health Association (1912) that a committee was established to report upon methods of examination and estimation of the numbers of colon bacilli found. The following abstract of the second report of this committee gives the recommendations for standard methods for bacteriological examination of shellfish

SEASONAL VARIATION IN THE BACTERIAL CONTENT OF OYSTERS

(GORHAM, 1912)

Date.	Average Total Bacterial Count of Shell Water of Five Oysters.	Proportion of Five Oysters Showing B. Coli in			Score.	B Coli Present in Water in	Temperature of Water C.
		1 c.c.	0.1 c.c.	0.01 c.c.			
BED NO. 8. PROVIDENCE RIVER							
Dec. 20, 1910	1000	3	1	0	4	0.01 c.c.	−1°
Jan. 14, 1911	750	5	3	1	41		
Jan. 25.....	80	4	3	0	23	0.01 c.c.	1°
Jan. 27.....	23	5	3	0	32		
Feb. 10.....	130	2	2	0	4	1.0 c.c.	0.1°
Feb. 28.....	140	0	0	0	0	0.0001 c.c.	1°
Mar. 11.....	200	5	4	0	41	0.01 c.c.	1.75°
April 14.....	275	5	2	0	23	0.01 c.c.	8.5°
April 28.....	700	5	5	4	410	0.0001 c.c.	12.5°
May 12.....	1700	5	5	5	500	0.0001 c.c.	15°
BED NO. 44. PROVIDENCE RIVER							
Jan. 7, 1911..	425	5	5	1	140	.....	0.25°
Feb. 10.....	250	4	0	0	4	.....	0°
Feb. 28.....	240	5	1	0	14	.....	0.5°
March 11....	100	5	2	0	23	.....	2°
April 14.....	210	2	0	0	2	.....	8.5°
April 28	1000	5	5	4	410	.....	11.75°
May 12.....	1100	5	5	4	410	.....	14.75°
BED NO. 204. WARREN RIVER							
Jan. 25, 1911	600	5	4	1	50	.....	0°
Feb. 10.....	140	0	0	0	0	1.0 c.c.	0°
Feb. 28.....	400	0	0	0	0	0.01 c.c.	0.75°
March 4.....	750	3*	3*	0*	*	.....	0.75°
March 11....	60	1	0	0	1	0.01 c.c.	3°
March 14....	3400	0	0	0	0	0.01 c.c.	8.75°
April 28.....	1050	5	5	4	410	0.01 c.c.	13°
BED NO. 205. WARREN RIVER							
Dec. 22, 1910	250	3	0	0	3	.....	−1°
Feb. 10, 1911	325	0	0	0	0	1.0 c.c.	0°
Feb. 28.....	450	4	2	0	14	0.01 c.c.	1°
March 4.....	600	2	2	1	5	.....	0.75°
March 11....	85	2	1	0	3	0.01 c.c.	2°
April 14.....	325	1	1	0	2	0.01 c.c.	8.25°
April 28.....	4000	5	5	5	500	0.01 c.c.	11.5°

* Only three oysters used.

which were adopted by the Association at its meeting in 1912:

Recommendations for Standard Methods for the Bacteriological Examination of Shellfish

Oysters in the Shell

Selection of Sample. Twelve (12) oysters of the average size of the lot under examination, with deep bowls, short lips, and shells tightly closed, shall be picked out by hand and prepared for transportation to the laboratory.

As complete a record of such data as is possible to obtain shall be made covering the following points:

The exact location of the bed from which the sample has been selected.

The depth of the water over the bed at time of collection.

The state of the tide.

The direction and velocity of the wind.

Other weather conditions.

The day and hour of the removal of the stock from the water.

The conditions under which the stock has been kept since removal from the water and prior to the taking of the sample.

The day and hour of the taking of the sample.

Transportation of the Sample. The oysters so selected shall be packed in suitable metal or pasteboard containers of such size and shape that a number of them can be enclosed in a shipping case capable of satisfactory refrigeration by means of ice. The important points in this connection are:

A. The prevention of the mixing of the oyster liquor of different samples, and of the mixing of the ice-water with the oysters.

B. The icing of the samples, if they are not to arrive at the point of laboratory examination inside of 36 hours, or if the outside temperature is above 50° F.

It is not necessary to enclose the oysters in an absolutely tight container, providing the above conditions are maintained.

Condition of Samples. Record shall be made of the general condition of the oysters when received, especially whether the shells are open or closed; of the presence of abnormal odors; and of the temperature of the stock.

Technical Procedure. The bacteriological examination shall be started as soon as possible after the receipt of the sample.

The oysters shall be thoroughly cleaned with a stiff brush and clean running water and then dried. The edges of the shell shall be passed through the flame or burned with alcohol.

The opening of the shell shall be accomplished by either of the following methods:

A. By the use of a sterile oyster knife in the usual manner.

B. By drilling through a flamed portion of the shell near the hinge with a sterile drill. The drill shall be sterilized, and the site of the operation on the shell shall be flamed at least once during the drilling process.

Bacterial Counts. Bacterial counts shall be made of a composite sample of each lot obtained by mixing the shell liquor of five oysters. Agar shall be used for the culture medium and in general the procedure shall be in accordance with the method recommended for the examination of water by the Committee on Standard Methods of Water Analysis of the American Public Health Association.

The water used for dilution purposes shall contain 1 per cent of sodium chloride, in order to approximate the natural salinity of oyster liquor.

The agar plates shall be incubated at 20° C. for three days and the colonies then counted.

Determination of Bacteria of the Bacillus Coli Group. The quantitative determination of the presence of B. coli shall be in accordance with the following procedure:

Measured quantities (1.0, 0.1, 0.01 c.c., etc., or their equivalents in dilutions) of the shell water of each of 5 oysters selected from the dozen, shall be placed in fermentation tubes containing lactose peptone bile, prepared according to the method recommended by the Committee on Standard Methods of Water Analysis. These shall be incubated for three days at 37° C., and the presence or absence of gas noted daily. For all ordinary purposes of routine work a development of from 10 to 85 per cent of gas during this time period shall constitute a positive test indicating a presumption of the presence of at least one bacterium of the Bacillus coli group in the quantity of shell water tested. But no final B. coli rating based on these results shall be used for official approval or condemnation unless positive confirmatory tests for the presence of organisms of the B. coli group shall have been obtained from the tube of highest or next highest dilution from each oyster, showing the presence of gas. These confirmatory tests shall be begun immediately upon noting the formation of gas, and carried out in accordance with the procedure recommended by the Committee on Standard Methods of Water Analysis.

Statement of Results. The results of the bacterial counts shall be expressed as Number of Bacteria per c.c. The results of the tests for B. coli shall be expressed either in the form of the following arbitrary numerical system to be known as "The American Public Health Association Method of Rating Oysters for B. coli; or in Estimated Number of Bacteria of the B. coli Group per c.c. of the Sample."

*The American Public Health Association Method of Rating Oysters for B. Coli.**

The following values shall be assigned to the presence of bacteria of the B. coli group in each of the 5 oysters examined, these figures being the reciprocals of the greatest dilutions in which the test for B. coli was positive:

If present in 1.0 c.c. but not in 0.1 c.c., a value of 1.
If present in 0.1 c.c. but not in 0.01 c.c., a value of 10.
If present in 0.01 c.c, but not in 0.001 c.c., a value of 100, etc.

The sum of these values for the 5 oysters gives the total value for the sample and this figure shall be taken as the "rating for B. coli."

The results shall be expressed in the following tabular form:

RESULTS OF TESTS FOR B. COLI IN DILUTIONS INDICATED

Oysters.	1.0 c.c.	0.1 c.c.	0.01 c.c.	Numerical Value.
1	+	+	o	10
2	+	+	o	10
3	+	o	o	1
4	+	o	o	1
5	+	o	o	1
		Total, or rating for B. coli		=23

+ = presence of bacteria of the B. coli group in fermentation tube test with lactose bile.

o = failure to demonstrate presence of bacteria of the B. coli group.

Estimated Number of B. Coli per c.c. If the standard B. coli rating above described is divided by 5, or, in general, if the rating is divided by the number of oysters tested, the result will be approximately the number of B. coli

* Where the term B. coli is used, it refers in all cases to bacteria of the B. coli group and not to the specific prototype.

per c.c. of shell water. Partly because it does not do this exactly, but also for simplicity and the avoidance of fractions, the method of stating results as an arbitrary rating is preferred by the committee. Practical experience with the method also has appeared to justify this preference.

Illustrations of the Application of the Method of Rating Oysters for B. Coli. Sometimes results similar to the following are obtained, that is, one or more oysters may show positive results in small quantities of shell water, while an equal number may show negative results in larger quantities. In this case the next lower numerical value shall be given to the positive results in the high dilutions, and such positive results shall be considered as being transferred to a lower dilution giving negative results in another oyster. This is done on the theory that inconsistent results, mathematically considered, may follow naturally from an unequal distribution of the bacteria in the shell water. This recession of the assigned values, however, shall not be carried beyond the point where the number of such recessions is greater than the number of instances where other oysters in the series failed to give positive B. coli results.

As examples of the method of obtaining the rating for B. coli, the following illustrations are given. They represent results that may be met with in practice:

CASE A.—RESULTS OF B. COLI TESTS IN DILUTIONS INDICATED

Oysters.	1.0 c.c.	0.1 c.c.	0.01 c.c.	Numerical Value.
1	+	+	0	10
2	+	+	0	10
3	+	+	0	10
4	+	0	0	10 (not 1)
5	+	+	+	10 (not 100)
				50 = rating

CASE B.—RESULTS OF B. COLI TESTS IN DILUTIONS INDICATED

Oysters.	1.0 c.c.	0.1 c.c.	0.01 c.c.	Numerical Value.
1	+	+	+	10 (not 100)
2	+	+	+	10 (not 100)
3	+	o	o	1
4	o	o	o	1 (not o)
5	o	o	o	1 (not o)
				23 = rating

CASE C.—RESULTS OF B. COLI TESTS IN DILUTIONS INDICATED

Oysters.	1.0 c.c.	0.1 c.c.	0.01 c.c.	Numerical Value.
1	+	+	o	10
2	+	+	o	10
3	+	+	+	100
4	+	+	+	10 (not 100)
5	+	o	o	10 (not 1)
				140 = rating

Oysters removed from the Shell (Opened or Shucked Stock).

Except as hereinafter stated, all the procedures and requirements for the examination of opened oysters, i.e., shucked stock, shall be those specified for the examination of oysters in the shell.

Selection and Preparation of Sample. The stock in the container from which the sample is to be taken shall be thoroughly mixed, and one or more wide-mouthed sterile jars of a total capacity of one quart shall be each half filled with the sample by means of a clean ladle or other instrument sterilized by flaming alcohol. The jar or jars shall be so sealed as to exclude all possibility of contamination from without.

Transportation of Samples. When the time between the collection of the sample and its examination exceeds 3 hours, or if the outside temperature is above 50° F., the sample shall be thoroughly refrigerated by means of ice placed around, but not in, the sample jars.

Technical Procedure. The bacteriological examination shall be begun as soon as possible after taking the sample. The sample shall be thoroughly shaken at least 25 times immediately before opening.

Bacterial Counts. The procedure specified for oysters in the shell shall be followed:

Determination of Bacteria of the Bacillus Coli Group. The procedure specified for oysters on the shell shall be followed, but attention is called to the fact that higher dilutions than $\frac{1}{100}$ c.c. are usually required. Triplicate fermentation tubes shall be inoculated from each dilution of the sample.

Statement of Results. The results of the bacteriological examination of the opened oysters, or shucked stock, shall be expressed in the same way as that specified for oysters in the shell, except that in the calculation of B. coli rating the values for the results of the positive fermentation tests after confirmation shall be recorded for each of the inoculations of each dilution. In order that the rating from these triplicate tests may be compared with that obtained from testing 5 oysters in the shell, the sum of the values for the triplicate tests shall be multiplied by $\frac{5}{3}$. If, instead, the sum is divided by 3, the result will give approximately the number of B. coli per c.c.

Clams and Other Shellfish

The methods for examining clams and shellfish other than oysters shall be those given above. Certain modifications are necessary in the method of handling the samples and the opening of the shells, etc.

Clams are more likely to lose water during transportation than oysters. It is therefore necessary to take greater precautions to separate different samples of clams from each other than in the case of oysters.

In opening soft clams it has been found that if two incisions are made through the mantle the shell water may be poured out without opening the shell.

Hard clams are more difficult to open, but if the shell be struck over the dorsal muscle with a small hammer an opening will be formed permitting the insertion of the knife to cut the muscle.

Sometimes clams and other shellfish contain too little liquor to make all of the tests above described. This is always the case when the shells are very small. Under these conditions the water from two or more shellfish shall be taken together and tested and considered as one.

Standards of Interpretation. As in the case of water it is neither practicable nor desirable to attempt to formulate any hard and fast standard for passing or condemning shellfish. It is very clear from the work carried out by the English Commission, and at Lawrence, Boston, Providence and New Haven in this country that shellfish from entirely unpolluted regions are free from colon bacilli and that the proportion of positive tests for these organisms increases with the increase in pollution. Just where to draw the line, however, it is not easy to say. According to Newlands and Ham (1910), the standards of permissible pollution adopted by various English and American workers vary from a positive test in 1 c.c. to a positive test in 0.1 c.c. of shell liquor. The Bureau of Chemistry of the United States Department of Agriculture condemns oysters sold in interstate commerce which show three positive tests out of five in 0.1 c.c. portions, and the same standard has been adopted by the Rhode Island Shell Fish Commission.

APPENDIX [1]

PREPARATION OF CULTURE MEDIA

In view of the marked influence upon bacteriological reactions of variations in culture media caused by differences both in ingredients and in technique of preparation, it is necessary that uniform methods be used in order to obtain comparable data. In specifying the various ingredients used in culture media it is the intention that they shall be uniform in quality, but it is not the intention that the recommendations as to ingredients and technical manipulations shall stand in the way of true progress as to improvements. When, however, improved or modified methods are used, the variations from the standard methods shall be plainly set forth together with the reasons for the modifications.

INGREDIENTS

Distilled water shall be used in the preparation of standard culture media.

Infusions of fresh lean meat, and not meat extract, shall be used as the basis of various media.

Peptone shall be that of Witte (dry from meat).

Gelatin shall be the best French brand, so called. It shall be as free as possible from acids and other impurities, and shall be of such a character that a 10 per cent solution

[1] From the Report of the Committee on Standard Methods of the American Public Health Association.

prepared in the usual way shall not soften when kept at a temperature of 25° C.

Commercial agar in threads shall be of as high a grade as can be obtained. Agar may be purified by washing.

The various sugars, such as dextr se, lactose, and saccharose, shall be as nearly as possible the chemically pure compounds designated. Unusual effort to obtain such sugars is considered to be necessary.

Glycerine shall be double distilled.

In place of litmus, a 1 per cent aqueous solution of Kahlbaum's azolitmin may be used.

Of the various other ingredients used, nearly all of which are of a mineral nature, special effort shall be made to see that they are chemically pure products within the full meaning of this expression.

STERILIZATION

Sterilization in the autoclave seems to be preferable to that in flowing steam. Both in the lowering of the melting point of gelatin and in the breaking down of sugar media the time of sterilization has a greater effect than does the temperature within the standard limits. It is, therefore, suggested that small containers be used and that media be sterilized in the autoclave at 120° C. (15 pounds pressure) for 15 minutes. A shorter period than this, in practice, sometimes results in incomplete sterilization, and a longer time results in the inversion of sugars or the lowering of the melting point of gelatin. Agar media should be melted before placing in the autoclave.

An important point in the sterilization of gelatin and sugar media is to have the sterilizer hot when the media are introduced, so that heating to the point of sterilization will be accomplished as quickly as possible. Also when sterilization is complete the media should be cooled rapidly. This not only reduces the time of heating, thus preserving

the gelatin and sugar, but also assists in the actual sterilization. It is also advisable in the use of the autoclave to keep the small pet cock at the bottom partially open so that steam is escaping during sterilization. This insures the removal of all air. If practicable, store media at room temperature for two days to see that sterilization is complete.

In intermittent sterilization, media shall be placed on each of three successive days in streaming steam for 30 minutes after the steam fills the sterilizer.

REACTION

Phenolphthalein shall be the standard indicator used in obtaining the reaction of all media. Turmeric paper possesses similar properties, and its use is advised where phenolphthalein is not available.

Titrations and adjustment of reactions shall be made as follows:

Put 5 c.c. of the medium to be tested into 45 c.c. distilled water. Boil briskly one minute. Add 1 c.c. of phenolphthalein solution (5 gm. of commercial salt in 1 liter of 50 per cent alcohol). Titrate while hot (preferably while boiling) with N/20 caustic soda. A faint but distinct pink color marks the true end-point. This distinct pink color may be more precisely described as a combination of 25 per cent of red (wave length approximately 658) with 75 per cent of white as shown by the disks of the color top (described under Record of Tints and Shades of Apparent Color, p. 10 of Standard Methods Report). In practice, titration is continued until the pink color of alkaline phenolphthalein matches that of the fused disks.

All reactions shall be expressed with reference to the phenolphthalein neutral point and shall be stated in percentages of normal acid or alkaline solutions required to neutralize them. Alkaline media shall be recorded with the minus (−) sign before the percentage of normal acid

needed for their neutralization, and acid media with the plus (+) sign before the percentage of normal alkaline solution necessary for their neutralization.

The standard reaction of culture media shall be +1.0 per cent. If it differs from 1 per cent by more than 0.2 per cent it should be readjusted.

Wherever reactions other than the standard above given are used it shall be clearly stated in all results of bacterial work, and the reasons therefor also stated.

STORAGE OF MEDIA

It is recognized by the committee that it is desirable to prepare media in large quantities in order to guard against discrepancies in composition; but, all things considered, the complications resulting from the varying amounts of heating incident to withdrawing portions from time to time and tubing it, are believed to more than offset this advantage. Consequently, when possible, media shall be put at once into tubes and placed in cold storage.

To guard against changes due to evaporation all media not used promptly shall be stored in a moist atmosphere, preferably in an ice-box, or else the flask shall be sealed by dipping the cotton plug in paraffin.

NUTRIENT BROTH

Nutrient broth shall be prepared as follows: Infuse 500 gm. chopped lean meat 24 hours with 1000 c.c. distilled water in refrigerator. Restore loss by evaporation. Strain infusion through cotton flannel. Add 1 per cent peptone. Warm on water bath, stirring until the peptone is dissolved. Heat over boiling water (or steam) bath 30 minutes. Restore loss by evaporation. Titrate. Adjust reaction to +1 per cent by adding normal hydrochloric acid or normal sodium hydrate, as required. Boil

2 minutes over free flame, constantly stirring. Restore loss by evaporation. Filter through absorbent cotton and cotton flannel, passing the liquid through until clear. Titrate and record final reaction. Tube, using 10 c.c. in each tube. Sterilize.

SUGAR BROTHS

Sugar broths shall be prepared in the same general manner as the standard nutrient broth, with the addition of 1 per cent of dextrose, lactose, saccharose or other sugar, just before sterilizing. The removal of muscle-sugar by inoculation with B. coli is unnecessary if small amounts of gas are disregarded.

If, however, test-tubes of small dimensions are used and the presence of any gas, however small, is taken to indicate gas formation the removal of muscle sugar is necessary.

The reaction of sugar broths shall be neutral to phenolphthalein.

Sterilization may be done in streaming steam, as usual, or in the autoclave at 120° C. (15 pounds pressure) for 15 minutes. Sterilization in the autoclave seems to be preferable to that in flowing steam.

For the routine work of testing samples in water for B. coli, especially large volumes of water are to be mixed with broths of such strength that the resulting mixture will be one of normal strength. Dextrose broth made with Liebig's Beef Extract is not equal in effectiveness to that made of fresh beef extract and should not be substituted for the latter.

LACTOSE BILE

The lactose bile medium consists of sterilized undiluted fresh ox gall (or a 10 per cent solution of dry fresh ox gall) to which has been added 1 per cent of peptone and 1 per cent of lactose. The addition of peptone is important.

GELATIN

No gelatin media should be employed having a melting point below 25° C. The percentage of gelatin added may be increased to bring the melting point up to the desired figure. With most gelatin on the market 11 per cent seems to be preferable to the standard of 10 per cent, provided the gelatin is weighed out without correcting for contained moisture, as appears to have been the custom. Ten per cent, or even 20 per cent of moisture commonly occurs in laboratory gelatin, and unless this is taken into account in weighing, the stiffness of the media is materially affected and the bacterial results obtained considerably modified. All gelatin should be tested for moisture before using by drying a sample for half an hour at 105° C. The stock should be kept under uniform conditions in tight containers, so that the percentage of water present may then be properly accounted for and the weight on a dry basis be used in making up the medium.

AGAR

For bacterial counts 10 gm. of agar per liter should be used. The smaller amount seems to be sufficient to carry the added water and the medium is less stiff. This appears to give higher and more consistent counts. Fifteen grams may be employed for keeping cultures.

North medium is especially valuable for keeping cultures, particularly cocci. This is composed as follows:

500 c.c. Extract of 500 gms. of chopped beef.
500 c.c. Distilled Water.
10 gm. Agar.
20 gm. Gelatin.
20 gm. Peptone.
5 gm. Sodium Chloride.
Reaction neutral.

It is well to determine the reaction of the media after sterilization, as during this process the reaction often changes

and the final results should correspond to the acidity recommended by the standard methods. What has been said regarding the necessity of correcting for moisture in gelatin applies with equal force to agar and for the same reasons.

NUTRIENT GELATIN AND AGAR

Nutrient gelatin and agar shall be prepared as follows:

	Gelatin.	Agar.
1.		Boil 15 gm. thread agar in 500 c.c. water for half an hour and make up weight to 500 gm. or digest for 15 minutes in the autoclave. Let this cool to about 60° C.
2.	Infuse 500 gm. lean meat 24 hours with 1000 c.c. of distilled water in refrigerator.	Infuse 500 gm. lean meat 24 hours with 500 c.c. of distilled water in refrigerator.
3.	Make up any loss by evaporation.	
4.	Strain infusion through cotton flannel.	
5.	Weigh filtered infusion.	
6.	Add 1 per cent Witte's peptone and 10 per cent Gold Label sheet gelatin on dry basis.	Add 2 per cent of Witte's peptone.
7.	Warm on water bath, stirring till peptone and gelatin are dissolved and not allowing the temperature to rise above 60° C.	
8.		To 500 gm. of the meat infusion add 500 c.c. of the 3 per cent agar, keeping the temperature below 60° C.
9.	Titrate, after boiling one minute to expel carbonic acid.	
10.	Adjust reaction to +1.0 per cent by adding normal hydrochloric acid or sodium hydrate as required.	
11.	Heat over boiling water (or steam) bath for 40 minutes.	
12.	Restore loss by evaporation.	
13.	Readjust to +1.0 per cent if necessary and boil 5 minutes over free flame, constantly stirring.	
14.	Make up loss by evaporation.	

15. Filter through absorbent cotton and cotton flannel, passing the filtrate through the filter until clear.
16. Titrate and record the final reaction.
17. Tube, using 10 c.c. of medium in each tube.
18. Sterilize 15 minutes in the autoclave at 120 degrees, or for 30 minutes in streaming steam on three successive days. Put the gelatin at once into ice-water till solidified.
19. Store in the ice-chest in a moist atmosphere, to prevent evaporation.

LACTOSE LITMUS AGAR

Lactose litmus agar shall be prepared in the same manner as nutrient agar, with the addition of 1 per cent of lactose to the medium just before sterilization. The reaction shall be made neutral to phenolphthalein (see p. 267).

If the medium is to be used in tubes the sterilized azolitmin solution shall not be added until just before the final sterilization.

If the medium is to be used in Petri dishes the sterilized azolitmin solution shall not be added to the medium until it is ready to be poured into the dishes.

More colonies and better general results are obtained on the lactose litmus agar plates, when the litmus and lactose are each sterilized separately and added to the plate with the neutral agar at the time of planting. Good results can, however, be obtained, if the agar and lactose are mixed and sterilized in an autoclave at 120° C. for 15 minutes only.

The azolitmin on the market varies considerably, much of that sold being entirely unreliable for the purpose. A 1 per cent solution of Kahlbaum's azolitmin, if boiled for 5 minutes, readily dissolves and needs no correction for acidity if added to standard agar. Many bacteriologists prefer pure litmus to azolitmin, and it is therefore suggested that its use be made optional. Both total and red colonies may be counted after from 18 to 24 hours

when incubated at 37° C. Such tests are sometimes used in the control of filtration plants.

LIVER BROTH

This medium is made from a hot infusion of beef liver instead of fresh meat, and is, in other respects, with the exception that phosphate is added the same as dextrose broth, but it is a richer food medium for bacteria. It gives gas formation with all species which ferment dextrose and develops attenuated bacteria, whether gas-forming or not, to a better degree than does beef broth. It is also especially suited to the rejuvenation of species in pure culture.

FORMULA

Beef Liver	500 gm.
Peptone (Witte's)	10 gm.
Dextrose	10 gm.
Di-Potassium Phosphate (K_2HPO_4)	1 gm.
Water	1000 gm.

1. Chop 500 gm. of beef liver into small pieces and add 1000 c.c. of distilled water. Weigh the infusion and container.

2. Boil slowly for 2 hours in a double boiler, starting cold and stirring occasionally.

3. Make up any loss in weight by evaporation and pass through a wire strainer.

4. To the filtrate add 10 gm. of peptone, 10 gm. of dextrose and 1 gm. of potassium phosphate.

5. After warming this mixture in a double boiler and stirring it for a few minutes to dissolve the ingredients, titrate with N/20 sodium hydrate, using phenolphthalein as an indicator, and neutralize with normal sodium hydrate.

6. Boil vigorously for 30 minutes in a double boiler, and 5 minutes over a free flame with constant stirring to prevent the caramelization of the dextrose.

7. Make up the loss in weight by evaporation and filter through cotton flannel and filter paper.

8. Tube and sterilize in an autoclave for 15 minutes at 120° C. (15 pounds).

Other valuable liver media (for use in the identification of B. sporogenes and other species) are prepared as given below:

LIVER GELATIN

1. Proceed as in steps 1, 2, 3, in preparing liver broth.

2. Cool the filtrate to 50° C. Add 10 per cent of sheet gelatin and stir a few minutes until dissolved.

3. Add 1 per cent of peptone, 1 per cent of dextrose and 0.1 per cent of potassium phosphate.

4. Stir until the ingredients are dissolved, keeping the temperature below 50° C., and then proceed as in steps 5, 6, 7, 8.

LIVER AGAR

1. Chop 500 gm. of beef liver into small pieces, add 500 c.c. of distilled water, and boil slowly for 2 hours, stirring occasionally.

2. Add 5 gm. of agar (dried at 105° C. for 30 minutes) to 500 c.c. of distilled water and digest for 30 minutes in an autoclave at 120° C. (15 pounds pressure).

3. After making up the loss by evaporation, pass the liver infusion through a wire strainer, add 500 c.c. of the filtrate to the agar solution and proceed as in steps 4, 5, 6, 7, 8, in preparing liver broth.

It is very important to note that liver broth should not be exposed to the high temperature attained in the autoclave any longer than 15 minutes, as prolonged heating above the boiling-point causes caramelization of the carbohydrates, rendering the medium less delicate for bacterial development. For the rejuvenation of attenuated cultures, especially B. sporogenes, the addition of very small pieces of liver tissue, which have been sterilized in Petri dishes

in the autoclave for 15 minutes improves the rejuvenating properties of the medium. They should be added to the tubes after sterilization.

Bacterial growth being very rapid in this medium, preliminary rejuvenation at 37° C. should be concluded between 6 and 12 hours.

HESSE AGAR

Agar (absolutely dry)	4.5 gm.
Peptone, Witte	10 gm.
Liebig's Extract of Beef	5 gm.
Salt	8.5 gm.
Distilled Water	1000 c.c.

Dissolve 4.5 gm. of dry agar in 500 c.c. distilled water by heating over a free flame, making up loss in weight by evaporation. Into another vessel 500 c.c. of distilled water is poured and to this is added 10 gm. of peptone, 5 gm. of Liebig's Beef Extract, and 8.5 gm. of salt. This is heated until all is dissolved and the loss in weight by evaporation is made up by adding distilled water.

Add the two solutions together; boil 30 minutes; make up loss in weight with distilled water, filter through absorbent cotton held in the funnel by cotton flannel, passing the filtrate through several times until perfectly clear. Test the reaction; adjust, if necessary, to +1.0 per cent normal acid, and tube, using 10 c.c. of medium in each tube. Sterilize for 15 minutes at 120° C. (15 pounds pressure) in an autoclave. Cool with running water and store in an ice-chest the air of which is saturated with moisture.

CONRADI-DRIGALSKI MEDIUM

These authors have modified lactose litmus agar by adding to it nutrose and crystal violet and by using 3 per cent of agar instead of 1 per cent. The crystal violet strongly inhibits the growth of many other bacteria, especially cocci, which would also color the medium red; the 3 per

cent agar makes the diffusion of the acid which is formed more difficult.

Three pounds of chopped beef are allowed to stand 24 hours with 2 liters of water. The meat infusion is boiled one hour and filtered. Twenty gm. of Witte's peptone, 20 gm. of nutrose, and 10 gm. of NaCl are then added, and the mixture boiled another hour. After filtration and the addition of 60 gm. of agar the mixture is boiled for 3 hours, made alkaline and filtered. In the meantime 300 c.c. of litmus solution (Kahlbaum) are boiled for 15 minutes with 30 gm. of lactose. Both solutions are then mixed and the mixture, which is now red, made faintly alkaline with 10 per cent soda solution. To this feebly alkaline mixture 4 c.c. of hot sterile 10 per cent soda solution are added and 20 c.c. of a sterile solution (1 to 1000) of crystal violet (Höchst B.).

ENDO MEDIUM

Make 3 per cent agar as follows:

1. To 1 liter of cold water add 30 gm. of powdered agar by sifting successive small portions upon the surface and allowing them to settle.

2. Add 10 gm. of Witte's peptone and 5 gm. of Liebig's meat extract.

3. Heat in double boiler until the ingredients are thoroughly dissolved.

4. Neutralize the sodium carbonate, using litmus as an indicator, and then add 10 c.c. of a 10 per cent solution of sodium carbonate.

5. Store the medium in flasks in lots of 100 c.c. or in larger known quantities, the flasks being large enough for the other ingredients to be added later.

6. Sterilize 2 hours in streaming steam.

It is essential to use powdered agar and cold water, since the agar settles more readily in cold water.

To use Endo medium: (*a*) make a 10 per cent solution of sodium sulphite in water; (*b*) make a 10 per cent solution of basic fuchsin in 96 per cent alcohol; (*c*) add 2 c.c. of the fuchsin solution to 10 c.c. of the sulphite solution, prepared as above, and steam a few minutes in an Arnold sterilizer.

7. Add 1 gm. of chemically pure lactose to each 100 c.c. of Endo medium.

8. Melt the Endo medium in streaming steam and add ½ c.c. of the fuchsin-sulphite solution described above.

9. Pour plates, and allow them to harden thoroughly in the incubator.

The lactose used must be chemically pure, and the sulphite solution must be made up fresh every day.

HISS' MEDIA

Two media are used: one for the isolation of the typhoid bacillus by plate culture, and one for the differentiation of the typhoid bacillus from all other forms in pure culture in tubes.

Plate Medium. The plate medium is composed of 10 gm. of agar, 25 gm. of gelatin, 5 gm. of sodium chloride, 5 gm. of Liebig's extract, 10 gm. of dextrose, and 1000 c.c. of water. When the agar is thoroughly melted the gelatin is added and completely dissolved by a few minutes' boiling. The medium is then titrated, to determine its reaction, phenolphthalein being used as an indicator. The requisite amount of normal hydrochloric acid or of sodium hydrate solution is added to bring it to the desired reaction; i.e., +2. To clear the medium add the whites of one or two eggs, well beaten in 25 c.c. of water, boil for 45 minutes, and filter through a thin filter of absorbent cotton. Add the dextrose after clearing. The reaction of the medium is most important; it should contain never less than 2 per cent of normal acid.

Tube Medium. The tube medium contains agar 5 gm., gelatin 80 gm., sodium chloride 5 gm., meat extract 5 gm., and dextrose 10 gm. to the liter of water; reaction +1.5. The mode of preparation is the same as for the plate medium, care being taken always to add the gelatin after the agar is thoroughly melted, so as not to alter this ingredient by prolonged exposure to high temperature. The dextrose is added after clearing.

MILK

The milk to be used as a culture medium shall be as fresh as possible, "Certified Milk" being ordinarily the best obtainable in city laboratories. It shall be placed in a refrigerator over night to allow the cream to rise and the suspended matter to settle. The skimmed milk shall be siphoned off into a flask for use. It will be found more convenient, however, to allow the milk to stand in a separatory funnel. Should the milk be too acid the reaction shall be corrected to +1 by the addition of normal sodium hydrate. It is then ready to be tubed and sterilized. Litmus milk shall be prepared as above, with the addition of sterile 1 per cent azolitmin. As it is impossible to make each lot of litmus milk with the same shade of color, it is recommended that a control tube be always exposed with the inoculated tubes for purposes of comparison.

NITRATE BROTH

Dissolve 1 gm. peptone in 1 liter of tap water, and add 0.2 gm. of nitrite-free potassium nitrate.

PEPTONE SOLUTION FOR INDOL TEST

Broth which has been inoculated with B. coli to remove the muscle sugar contains toxins which interfere with the proper growth of many species, hence peptone solution,

(1 per cent peptone in water) is recommended for use in the indol test.

ÆSCULIN MEDIA

Broth. Dissolve 10 gm. peptone, 5 gm. commercial sodium taurocholate (or glycocholate), 0.1 gm. æsculin and 0.5 gm. soluble iron citrate in 1 liter distilled water.

Tube in 5 c.c. quantities in test-tubes and sterilize.

It is preferable to keep the iron citrate solution separate and to add it to the rest of the media as used, since the iron causes a precipitate. Sometimes the iron citrate will not readily dissolve. In such case a few drops of ammonia will cause immediate solution, especially on warming. The excess of ammonia should then be boiled off.

Agar. This medium is made in the same manner as the broth with the addition of the usual amount of agar.

APPARATUS

Few definite requirements need be made respecting apparatus. The quality of the glass shall be such as not to be easily acted upon by the reagents used, and all glassware shall be scrupulously clean when used. When necessary it shall be sterilized by dry heat for one hour at about 150° C. A slight browning of the cotton stoppers is a good index of proper exposure.

In some operations, as for example, the determination of the thermal death point, it is necessary to use test-tubes of a definite size and thickness. For this purpose the standard size culture tube shall be 15 cm. long, 1.6 cm. in diameter, and of medium weight. Tubes to be filled with gelatin for quantitative work may be those described as 6 in. $\times \frac{5}{8}$ in. "heavy."

The standard loop for making transfers shall be prepared as follows:

Bend the end of a piece of No. 27 platinum wire about 10 cm. long over a bit of No. 10 wire, and fasten the loop thus formed into a glass rod to serve as a handle. A loopful of culture shall be interpreted as meaning all the fluid that the loop can hold. That is, the fluid shall form a bi-convex body and shall not be simply a film covering the space in the loop.

The standard fermentation tube shall be a tube 1.5 cm. in diameter, bent at an acute angle, closed at one end and provided with a bulb at the other which is large enough to receive all the liquid contained in the closed portion. The length of the closed end of the tube shall be about 14 cm. The bulb shall have a diameter of about 3.8 cm.

INCUBATION

There shall be two standard temperatures of incubation—20° C. and 37° C., the first corresponding to ordinary room temperature, the second to blood heat. The temperature of the incubators shall not be allowed to vary from these two standards by more than 1° C. in either direction.

The atmosphere of the incubator shall be kept moist, preferably near the point of saturation. The incubator shall be ventilated so as to insure a reasonably good circulation of air in order to prevent the accumulation in the incubator of gases which might be prejudicial to the development of the bacteria.

No definite period of incubation can be prescribed which will be suitable for all the work of species determination, but in reporting results the period used shall always be stated and form a part of the report. General statements as to the necessary periods will be found in connection with the principal tests.

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